

THE AMERICAN JOURNAL OF PHYSIOLOGY

VOLUME 152

JANUARY-MARCH 1948

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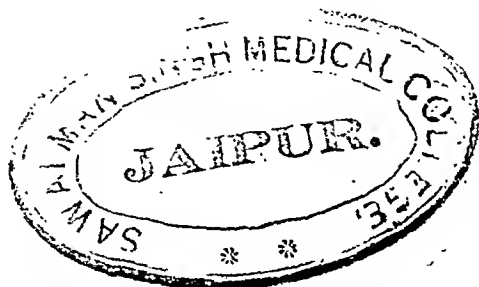
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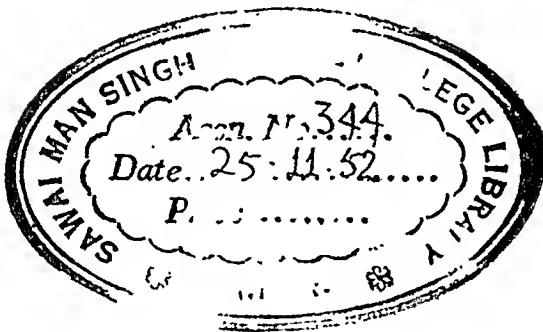


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Washington, D. C.

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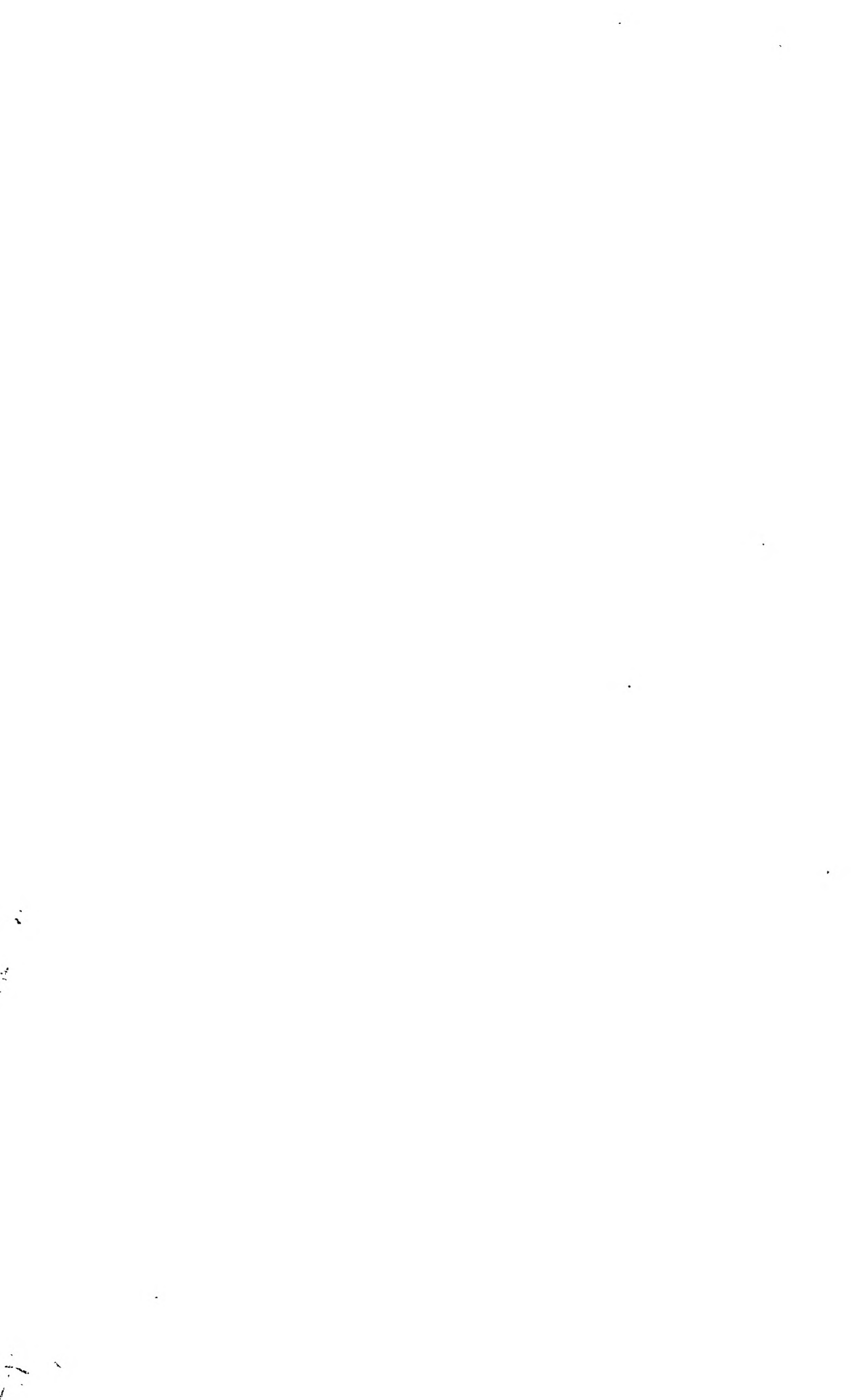
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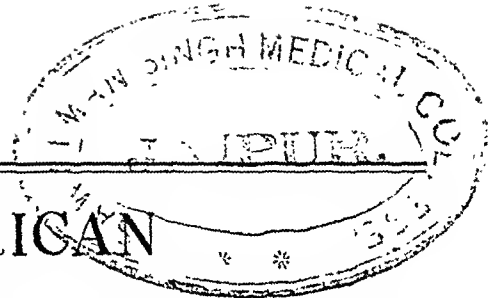
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THE AMERICAN JOURNAL OF PHYSIOLOGY

Published by
THE AMERICAN PHYSIOLOGICAL SOCIETY

VOLUME 152

FEBRUARY 1, 1948

NUMBER 2

NEURAL RESPONSES AND REACTIONS OF THE HEART OF A HUMAN EMBRYO¹

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Received for publication December 5, 1947

The observations recorded in this paper were made on the isolated heart of a human embryo of 100 millimeter crown-rump length. Such a size on the basis of age-length curves (1) would indicate a presumptive fertilization age of 13 weeks. The isolated ventricles of the heart, after removal in preparation for experiments, were nine millimeters from base to apex.

The immediate object of the study was to determine whether cholinergic inhibitory nerves (vagi) would function at this embryonic stage and to compare other functional characteristics with those accredited to adult mammalian hearts.

The observations followed a procedure which has been used to advantage by the authors in unpublished studies on the hearts of several mammalian forms. It consists simply of initial rapid chilling of the heart, in a modified Ringer's solution, to about 5°C. and the subsequent study of cardiac strips at temperatures lower than the normal body temperature. In such a cold solution mammalian cardiac tissue will remain quiescent but viable often as long as four days; strips of either atrium or ventricle will beat spontaneously when the temperature is raised, and at room temperature (e.g., 24°C.) the beats are more vigorous than at body temperature. When adequately oxygenated the beats will continue for hours, and such strips have been used on three successive days after cooling to 5°C. during the night.

The embryonic heart was divided into three preparations, the sinus-atrium and two ventricular portions, the dissection being completed in the cold Ringer's solution. Records of contractions were made by the simplest suspension

¹ This investigation was aided by a grant from the American Medical Association.

method. For recording, the preparation was attached to a light magnesium ribbon lever and immersed in 50 cc. of saline solution, through which oxygen was gently bubbled. The buffers of the solution maintained a pH of 7.5 to 7.6 throughout all observations. The test for vagal inhibition was that described for cold-blooded hearts by Garrey and Chastain (2). After eserizing, acetylcholine was added to a desired concentration to produce stimulation of the inhibitory nerves. The inhibitory action of the vagi on the pacemaker and atrial contraction of mammalian hearts is strikingly effective by this method of testing, and effects are obtained at low temperatures as well as at body temperature, hence its use in the present investigation.

Ventricles. The right ventricular muscle was mounted for recording and gradually warmed from 5°C. to room temperature (24°C.). After a half hour the preparation contracted in response to electrical stimulation and an occasional automatic beat was observed. These contractions were not weakened by eserine (1-20,000) plus acetylcholine bromide in concentrations up to one part in 10,000 of saline. After repeated washing with Ringer's solution, the ventricular muscle was subjected to a continuous bath of one in one million acetylcholine bromide. In this solution it responded to induction shocks and a regular rhythm developed. These spontaneous beats appeared at first in groups which changed in such a way as to indicate the disappearance of partial block between the point of impulse initiation and the main muscular mass. Once established the beats continued with force and regularity at a rate of 17 per minute at 24°C. Thus the presence of acetylcholine did not, through any inhibitory action, prevent the development or conduction of autogenous contractions. Two hours later the heart was beating strongly; then, following further treatment with eserine (1-50,000), the concentration of acetylcholine was raised to one in 100,000 and finally to one in 25,000 without any evidence of inhibition of rate or force of contractions (fig. 1).

Adrenaline chloride (one in 250,000) failed to elicit significant effects beyond a slight acceleration of rate. It was not determined whether this was due directly to a muscular action or indirectly to the influence of functionally active sympathetic nerves. Identical reactions were obtained from our second ventricular preparation, the contractions of which were at the identical rate of the first preparation, a rate less than one-third that of the sinus-auricular preparation. It would thus appear that the rhythmogenic characteristics and ratios which are found in different parts of the heart of the adult are already established prior to the age of this embryo. Histological preparations revealed the presence of many nerve fibers throughout the ventricles; nevertheless, there was a complete absence of any cholinergic inhibitory effects. This, however, is not surprising since, by use of this same method of testing, we have been unable to demonstrate cholinergic inhibition of the adult ventricular muscle of any of the many types of mammals studied.

Sinus-atrium. Three hours after its initial cooling the auricular preparation was mounted for recording at room temperature. The beats gradually increased to a uniform height and continued at a rate of about 63 per minute. Unlike the ventricles this atrial preparation did show definite, though slight, evidences of

inhibitory responses when the acetylcholine tests of vagal inhibition were applied, but the sensitivity was low. For example, after anticholinesterase treatment with eserine (one in 50,000) this preparation did not react in any detectable way to acetylcholine bromide unless the concentration was greater than one part in 10 million, which caused a 20 per cent reduction in the height of contraction followed by a partial block between the sinus (pacemaker) and the atrium, resulting in a sudden drop of atrial contractions to one-half the pre-existing rate. The

FIG. 1

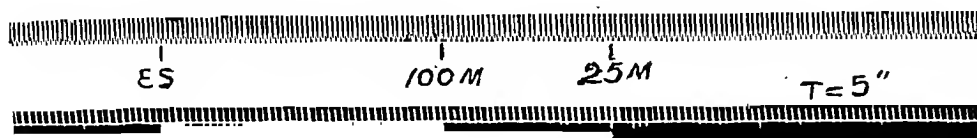


FIG. 2

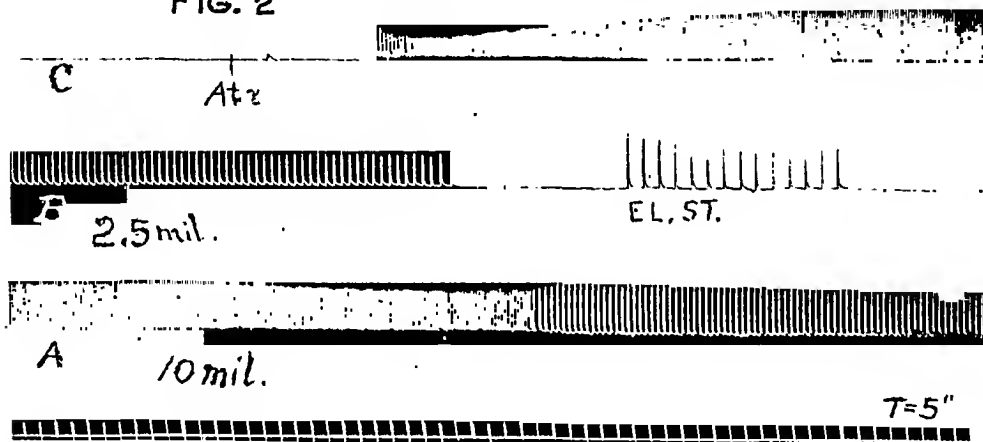


FIG. 1. Automatic beats of the ventricle. Not inhibited by eserine (ES, 1/50000) plus acetylcholine one in 100,000 (at 100 M) or one in 25,000 (at 25 M). Time tracing = 5" intervals.

FIG. 2. Sino-atrial beats. A, B and C are successive records. A records the long latency and 2/1 block produced by acetylcholine (one in ten million, 10 mil.). B records the complete s/a block produced by stronger acetylcholine solution (one in 2.5 million, 2.5 mil.), but contractions were elicited by electrical stimuli. C shows the release from acetylcholine action when atropine, one in one million, was added at atr. The same time record (T = 5") applies to all tracings.

latent period of the drug action was very long, for the partial block did not appear until nearly two minutes after application of the acetylcholine. At this juncture the concentration of acetylcholine was quadrupled (one in 2.5 million). Again after a latent period of nearly two minutes the partial block suddenly became complete and the atria remained quiescent. Inhibition, however, was far from complete since they responded with contractions upon electrical stimulation. Release from the effects of acetylcholine followed 45 seconds after treatment with atropine (one in one million); the block disappeared, the rate accelerated

promptly to the normal rate and the height of contractions which had been weakened to one fourth of their previous height progressively increased until they exceeded the norm by about 30 per cent (fig. 2).

Since atropine acts by paralyzing vagal action, these results are consistent with the interpretation that at this embryonic stage inhibitory function of the vagus is already demonstrable on the sinus rate (slight), on conduction and on the strength of atrial contraction, but the inhibition is slight indeed compared with that which the authors have obtained by the same method when applied to adult atria of several mammalian forms. In the adult heart inhibition results from extreme dilutions of acetylcholine, e.g., one part in 300 million. There is practically no latency and the weakening of the contractions is profound. Nerve cells and fibers were abundant in the atrial preparation of our embryo, yet the cholinergic inhibitory responses were feeble and their character and course was such as to suggest the possibility that at this early embryonic stage the inhibitory nerves function weakly and that their full functional potentialities are acquired only at a later stage of development. Such a delay in the development of its inhibitory action by the vagus of the chick has been reported by Pickering (3), and Barcroft found that vagal inhibition of the heart of the foetal lamb could be demonstrated only after the 88th day of gestation (cf. Barclay, Franklin and Prichard, 4).

In tests made for us by Dr. Charles E. King it was found that the intestinal muscles of this embryo responded promptly and vigorously to acetylcholine. The functional responses of the gastrointestinal tract were developed at this stage far in excess of any demonstrable in our cardiac tests.

Temperature reactions. The incidental observations on the effects of temperature referred to above were extended to include the entire gamut from 3° C. to 40° C., thus adding a record of the reactions of a human embryo of a specific age to the voluminous literature in this general field of temperature effects.

The sino-atrial preparation beat most vigorously at a temperature between 25° and 30°C. When cooled to 20° and then to below 15°, the preparation maintained vigorous beats at progressively slower rates. At 10° the atrial contractions suddenly ceased, although effective responses were elicited by electrical stimuli even at 3°C. These results suggest that temperatures below 10° acted primarily by blocking sino-atrial conduction, an interpretation which was further supported by the fact that upon warming the preparation to 12° the atrial contractions reappeared suddenly with full vigor and with complete regularity, as if block had suddenly ceased. Such a result calls for tests for impulse formation in the sinus while the atrium is at rest below 10°C., tests which were not feasible during our experiments.

A graph in which rate of beat is plotted against temperature is shown in figure 3. It shows the slow rate at low temperature, deviating little from a straight line between 20° and 35°. Below 20° there is a definite deflection, giving the lower part of the well-known S-shaped curve so characteristic of the action of temperature on rhythmic processes. Temperature coefficient, calculated from the data of the graph, was greater than five for the ten degrees

change in rate between 10° and 20° , while between 30° and 40° it was only 1.6. For the rectilinear portion of the graph the rate varied about seven beats for each centigrade degree.

At the normal body temperature of 37°C . this isolated embryonic heart beat at the rate of 157 per minute, which may be considered the fundamental pace-maker rate of the preparation. It is not without interest to note that the average mid-foetal heart rate is given as 156 per minute (Windle, 5) and that this intrinsic

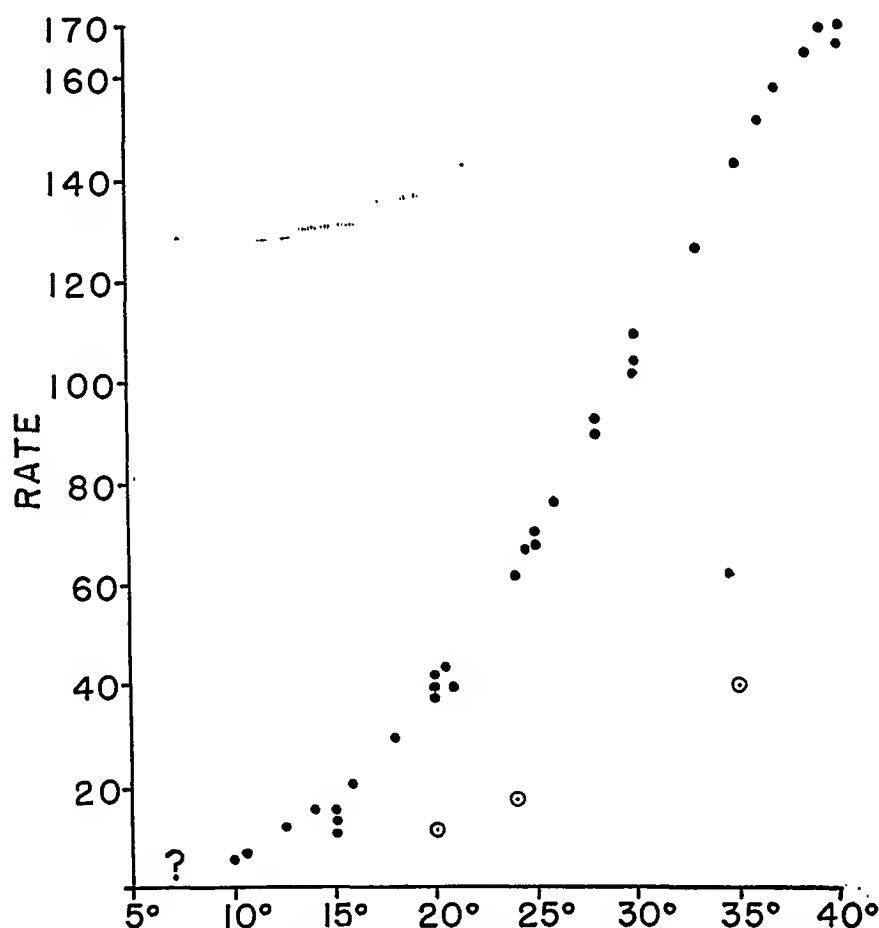


FIG. 3. Heart rates (*ordinates*) are plotted against temperatures (*abscissae*). Solid dots represent rates of the sinus-atrium. The three circles indicate ventricular rates.

rate progressively slows to the time of birth, due presumably to the development of vagal tone during the later stages of foetal life and to its inhibitory impress which maintains the slower rate throughout life. With removal of vagal influences either by section of the nerves or by atropinization, as is well known, the release from tonic inhibition reestablishes the rapid rate which approximates the fundamental rhythm of the embryonic heart prior to the development of vagal inhibitory tone.

In the case of the beating ventricular preparation at 24° the rate was 17 per

minute as compared with a sino-atrial rate of 62, giving a ratio of one to 3.6. Observations were made at only two other temperatures; at 20° the ventricular rate was 12 while that of the atrium was 40 per minute and at 35° the ventricular rate was 39 per minute while that of the atrium was 142 per minute. The ratios in the two instances were 3.3 and 3.6, respectively. It would appear from these figures that the rhythmogenic characteristics of sino-atrium and ventricle include an intrinsic rate ratio not far from 3.5/1, that this relationship is established prior to the age of our embryo (13 weeks) and corresponds in general to the status known to exist in adult hearts.

SUMMARY

1. Isolated and previously chilled preparations of the atrium and of the ventricles of a 13-weeks human embryo beat for hours at room temperature in well oxygenated Ringer's solution.

2. Acetylcholine even in high concentration had no inhibiting effects on the ventricles.

3. There was some inhibitory action of acetylcholine on the sinus-atrium. The effects were produced only by strong solutions and were delayed in their onset even one or two minutes. Profound inhibition, such as the authors have seen with other postpartum mammalian hearts, did not occur.

4. The results suggest that the full inhibitory function of cardiac nerves is a later foetal development.

5. The isolated sino-atrial preparation beat at all temperatures between 10°C. and 40°. The intrinsic sinus rate at 37°C. was 157 per minute. The ratio of this rate to the slower idioventricular rate was about 3.5/1. These rates approximate those of the adult heart when free of nervous regulation and indicate that the rhythmicity 'pattern' of the heart is established early in embryonic life.

We are indebted to Dr. Mary E. Gray for the histological examination of this heart, prepared with Ranson's pyridine silver stain. We summarize her findings as follows: Many large nerve trunks following blood vessels in both auricles and ventricles. Many fine nerve branches among the muscle cells of the entire heart. Numerous ganglion cells in the subepicardial connective tissue of the auricles only. No ganglion cells were found in the ventricles.

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EXPERIMENTAL HYPOTHERMIA AND REWARMING IN THE DOG RECOVERY AFTER SEVERE REDUCTION IN BODY TEMPERATURE¹

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Received for publication January 7, 1948

In studies of the hypothermic state, observations have been made upon the capacity for recovery following rather drastic reduction of body temperature, and certain points of interest are incorporated in the present report. It is noteworthy particularly in view of the degree of cooling which can be sustained under certain conditions with the sequel of a complete recovery.

METHODS

Dogs of an unselected but generally healthy stock were used. The procedure followed consisted simply in submerging the undepilated³ and lightly anesthetized preparation to the neck in a bath of iced water; immersion was continued until a crisis, respiratory or cardiac, supervened. In some instances, following partial rewarming, animals were subjected to re-immersion. While recourse to anesthetics in this type of experiment is difficult of defense on a strictly physiological basis (1) it proved desirable from certain standpoints to induce an initial narcosis sufficient at least to offset the intense psychomotor disturbances otherwise incurred by abrupt immersion in a chilled bath. Moreover, light anesthesia afforded an initial facilitation of reduction in temperature which is otherwise very difficult to achieve (2). Accordingly, an anesthetic agent of short-acting nature was used—either pentothal sodium *per venam* in sedative dosage, pentothal supplemented with ether, or ether alone, withdrawn as soon as feasible. In the final experiments cyclopropane was employed, and proved most satisfactory of all. During immersion (bath temperatures ranging from 2° to 9°C) records were kept of pulse, respiration, deep rectal temperature (4–6 cm.) and, occasionally, deep visceral and muscle temperature. Periodic electrocardiograms were recorded.

The immersion period ranged from 67 to 193 minutes (avg. 155); sudden collapse of pulse or of respiration signalled the institution of rewarming measures. Artificial respiration was occasionally required. Rewarming was carried out routinely by immersion in water at 42° to 45°C.

¹ This work was carried out under Contract W33-038 ac 14757 with the Aeromedical Laboratory, Air Materiel Command, A.A.F., Wright Field, Dayton, Ohio.

² Now in the Department of Pharmacology.

³ A series of trials failed to demonstrate an appreciable influence of depilation upon rate of cooling. This is in agreement with an observation by Speakman (2).

RESULTS

Thirteen animals sustained reduction in rectal temperatures to an average of 14.9°C. (range 11.7° to 16.8°C.). These are listed in table 1. Recovery in each

TABLE 1. SUMMARY OF CASES SURVIVING ACUTE HYPOTHERMIA

ANIMAL NO.	LOWEST RECTAL T°C.	REMARKS
1	16.4	Pulse 28, irregular; resp. 12, shallow. Hot water immersion; rapid recovery
2	15.0	Pulse and resp. ceased; artificial resp. and massage, resp. and pulse resumed in 5 min. Recovery. Shivering at 29°
3	15.2	No P wave in EKG. Hot water; shivering at 23.8; good recovery
4	13.0	Pulse 18, resp. 6, irregular. Rapid rewarming; shivering at 17°, pulse 72. Recovery
5	15.0	Pulse 18-20; irregular; rewarming; movements and whimpering at 24°. Rapid recovery.
6	16.0	Pulse 32, weak, irregular; artificial resp.; rewarming with immediate response; shivering at 20°
7	15.0	Pulse 2-3; resp. 4, shallow; rewarming, pulse rose to 20 before rectal t° responded; shivering at 26°
8	14.8	Pulse 24, irregular, resp. 6; rewarming; shivering at 15°. Rapid recovery.
9	15.0	Resp. 3, pulse impalpable; rewarming; at 24° voluntary movements. Good recovery.
10	14.2	Pulse 30, irregular; rewarming; shivering at 18.8; pulse 100. Recovery.
11	15.6	Pulse 32, resp. 8; rapid recovery on rewarming
12	11.7	Cyclopropane anesthesia; rewarming; knee jerk + ⁺ rectal t° 17°
13	16.8	Pulse 16, irregular; spontaneous rewarming; rectal t° 20° in 120 minutes, pulse 58. Good recovery

instance was complete, and the animals were returned to quarters within two hours of the immersion period, apparently little the worse for the experience. In two animals the rectal temperature was reduced to 15°C. or below on two occasions with an interlude of one week.

Shivering appeared to exert no marked influence upon the rate of temperature fall, which was essentially linear (figs. 1, 2, 3). In the downward course, shivering began variously at rectal temperatures of 30° to 35.8°C.; during rewarming its onset occurred over a wide spread—ranging from 15°C. in one instance (Expt. 8) to 29°C. (Expt. 2). It varied widely in intensity and did not seem significantly to accelerate recovery.

Skeletal muscle tonus, greatly exaggerated during the cooling process, was not detectable below rectal temperatures of 20° to 18°C. and the intense rigidity characteristic of the cooling animal was succeeded by a flaccid state, in agreement with the observations of Crismon (3). In the rewarming process, tonus reappeared within the same temperature range, with recovery of superficial reflexes.

Eight animals failed to respond to warming, or succumbed before resuscitation measures were started. The cause of death in some cases appeared to be circula-

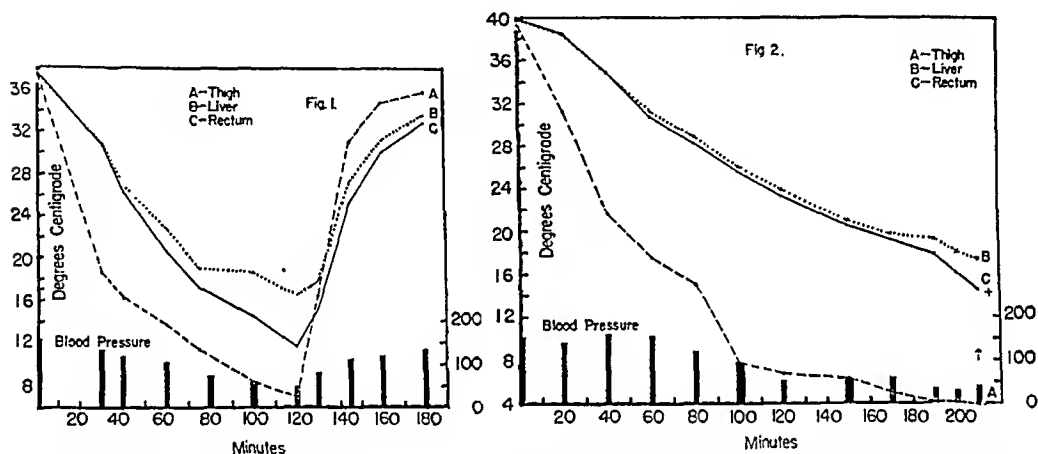


FIG. 1. EXPT. 12. 8.1 kgm. Cyclopropane anesthesia. Rewarming begun at 120 minutes. Automatic recording of temperature in this and other figures. Carotid blood pressure by photoelectrokymographic registration.

FIG. 2. EXPT. 18. 10.1 kgm. Pentothal sodium anesthesia. Removed from bath at ↑. Exitus at +.

tory failure of cardiac origin. Associated with the marked degree of bradycardia which supervened as rectal temperatures fell below 20°C., engorgement of the heart and great veins was a consistent autopsy finding.

The reliability of rectal readings as an absolute index of mean body temperature under conditions of immersion has, of course, been questioned (4) and it is to be expected that a deep-to-surface gradient exists. This is shown in the record of the hypothermia in one experiment which is incorporated in figure 1. The temperatures of three body regions (liver, rectum, thigh muscle) as obtained by thermocouple needles were recorded automatically⁴. It is seen, in general, that the liver retained the highest temperature during the cooling process with, in this case, a maximal liver-rectum gradient of 4.8°C. The graph illustrates as well the linear fall characteristic of mean blood pressure as the hypothermic state

⁴ 'Speedomax' recorder, Leeds & Northrup Company, Philadelphia.

progresses. This is true as well of the pulse rate. In figure 2 is depicted the decline in temperatures and in blood pressure in an experiment in which, despite

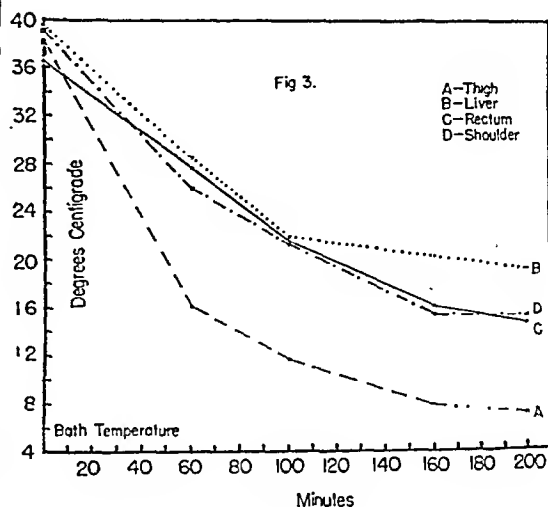
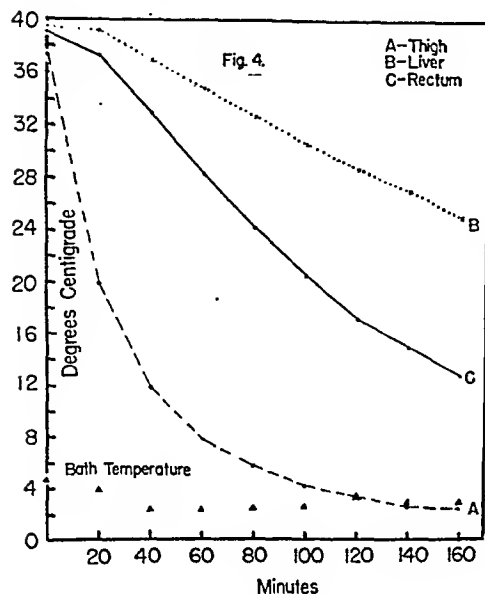


FIG. 3. AVERAGE OF THE REDUCTION IN TEMPERATURES in twelve experiments, for body regions as indicated. Average bath temperature 5.6°C .

FIG. 4. TEMPERATURE RECORDS in freshly sacrificed animal (11 kgm.) placed in iced water bath, showing marked lag in 'deep-to-surface' temperature gradient in the absence of circulation.

TABLE 2. DOG #14, FEMALE MONGREL, 5.5 KG.M.; ETHER ANESTHESIA, ATROPINE 5 MG.M. S. C.

TIME	RECTAL T°	REMARKS
0 min	38.8	Into bath at 4.5°
15 "	34.0	Slight shivering
35 "	26.0	Some muscular movements
55 "	19.0	Respiration 50
85 "	16.8	Pulse 22, irregular
110 "	15.0	Removed from bath
120 "	13.5	Respiration 14, pulse 18
135 "	12.5	Respiration 6
150 "	11.5	Rewarming begun: pulse 0., resp. 2
153 "	11.3	
164 "	14.5	Shivering begun
167 "	18.0	Respiration improving; knee jerk +
171 "	18.2	Respiratory gasps, followed by apnea, pulse feeble. Artificial respiration. No recovery

P.M. (173) min. Right auricle beating rhythmically; ventricle insensitive to irritation, enormously engorged. Lungs clear, viscera congested. No ascitic fluid.

a slower rate of fall and a smaller liver-rectum gradient (maximum 2.9°C .), recovery was not effected; death supervened five minutes after the rewarming

was begun. The data of twelve experiments on comparative temperature readings are summarized in figure 3.

One clearly defined instance of 'rewarming death' was recorded, an abbreviated protocol for which is given in table 2. At a rectal temperature of 11.5°C. the pulse, noted a moment previously at eight per minute, temporarily ceased to register on the EKG recorder. Rewarming with artificial respiration was begun; a pulse reappeared, and in eleven minutes the rectal temperature had risen to 14.5°C., at which point shivering commenced. Seventeen minutes after rewarming was begun the rectal reading was 18°C., respiration 18, knee jerk positive. Twenty-one minutes after the start of resuscitation (rectal 18.2°C.) the animal began gasping, the pulse became feeble and erratic. Artificial respiration proved ineffective. This type of collapse has been noted (5, 6) and has been attributed to the overloading of the myocardium under conditions of hypoxia.

Electrocardiographic responses to progressive cooling will be discussed elsewhere. It is of interest to note, however, the remarkable slowing to which the cardiac cycle may be subjected. Cycles, for example, of 1.8 seconds have been recorded, the beats separated by extremely long diastolic pauses, with pulse rates as low as six per minute. Neither section of the vagi in the neck, nor atropinization appear to influence appreciably the course of the cardiac cycle under conditions of cooling. This is in accord with the findings of Grosse-Brockhoff and Schoedel (7).

DISCUSSION

Observations upon reversibility of hypothermic changes have been reported by a number of investigators. Britton (8), in one of the more comprehensive of earlier papers, remarked that cats, anesthetized with a chloroform-ether mixture and cooled in an air-chamber, 'may survive' cooling to a deep rectal temperature of 16°C.; recovery occurred more commonly if body temperature was not lowered below 20°C. Britton listed also the chronological reappearance of reflexes and reactions during the spontaneous rewarming process. More recently Crismon (3) and Spealman (2) have contributed pertinent observations. The former reported that application of warm water was rapidly effective in restoring the dog, provided either *a*) the circulatory and respiratory depression had not progressed beyond a certain point or *b*) the duration of hypothermia was restricted within rather definite limits. The tolerable hypothermia was not so severe in degree as that reported here. However, pentobarbital sodium was used as a pre-immersion anesthetic agent; in our experiment this barbiturate curtails the ability of the animal to withstand cold. In Crismon's experiments, for example, dogs were revived from rectal temperature levels below 24°C. only if permitted below this level for less than 20 minutes. In the present experiments animals have survived reduction to 14°, 15° and 16°C. rectal temperatures under circumstances in which the temperature was less than 24°C. for as much as 110 minutes (e.g., Expt. 10).

The use of substances such as pentobarbital sodium places the animal, in effect, in 'double jeopardy', for upon the depression induced by the initial anes

thesia is superimposed the narcosis of hypothermia, and the organism has therefore the combined effects of the two to overcome. Pentothal sodium was used in our experiments on the assumption that as cooling progressed the effects were supplanted by, and not combined with, cold narcosis. Admittedly, the time required for the influence of pentothal to subside under cooling is conjectural; and estimates in this case may possibly be overly sanguine. However, animals administered cyclopropane behaved identically, and administration was required only until a temperature of 27° to 25°C. had been reached; beyond this point, cold narcosis sufficed and there is at present no reason to believe that the transitory depressant effects of cyclopropane, even under conditions of cold, are not rapidly dissipated with the blowing off of the agent.

Spealman utilized animals without benefit of anesthesia and found it difficult to obtain a marked hypothermia. Large dogs (averaging 27.6 kgm.), for example, did not cool appreciably in baths at 20°C. (68°F.) or above; at 15°C. (59°F.) and below, cooling could occur, but in some animals a normal temperature level was maintained for at least five hours in a bath at 0°C. 'Serious impairment' was shown when rectal temperatures did decline to about 27°C. The linear fall in rectal temperature, as reported by Crismon (3) and in the present report was due, in all probability, to the influence of the initial anesthesia in vitiating the capacity to combat increased heat loss (1). It seems a fair surmise that if Spealman's conscious animals could have been cooled to the stage of cold narcosis, the rate of cooling from that point on would have been a linear one. Indeed, the anesthetized animal differs but little from the dead animal so far as rectal cooling (from an initial 37°C. or above) in a cold bath is concerned; two experiments in which freshly sacrificed animals were immersed in iced water revealed a linear rate of heat loss comparable in point of time to that which characterized the living, but anesthetized, preparation. An understandable lag occurred in decline in visceral temperature, in view of the absence of circulation (fig. 4).

Species other than the dog have been examined as to viability under the stress of sharply reduced environmental temperatures, and species differences, as usual, have been revealed. Simpson and Herring (9) observed that temperatures below 16°C. (in air) were lethal for the cat, a finding confirmed by Britton in 1922. Hamilton (10) found that unanesthetized rats—which cool appreciably even in water at 30°C., according to Spealman—did not survive below an internal temperature of 12°C. (54°F.); life was 'precarious' up to 16°C., indeed, and for survival the use of artificial respiration often was required. In cats and kittens a colonic temperature of 75°F. (24°C) was reported as not critical but a reduction to 68°F. (18.5°C.) was definitely inimical. Ware, Hill and Schultz (11) very recently reported that in their cold-chamber experiments, newborn rats have recovered from colonic temperatures of 5°C.⁵, 30-day old rats from 8°C., 60-day animals from 11°C. and 80-day old and up from 15°C. Two points of interest, especially, appear in their report: struggling only momentarily retarded rate of

⁵ Lutz (*Zeitschr. f. Kreislaufforsch.* 36: 625, 1944) reported recovery in the unanesthetized guinea pig from rectal temperatures of 3°C. by rapid warming in water at 50°C., with simultaneous artificial respiration and electrical stimulation of the heart.

fall (room temperature 2° to $4^{\circ}\text{C}.$), and if respiration were impeded the fall was accelerated. Meader and Marshall (12) cooled mice in a dry-ice chamber at an ambient temperature of $-10^{\circ}\text{C}.$, with exposures of 25 and 65 minutes. Temperature decline was linear in character, and internal readings of $14.5^{\circ}\text{C}.$, and as low as $8.5^{\circ}\text{C}.$, were recorded. Some of the animals recovered spontaneously, others required artificial heat.

Ariel, Bishop and Warren (13) reported that rabbits (anesthetized) could withstand reduction in iced bath to $20^{\circ}\text{C}.$ with spontaneous recovery, provided the reduction was not too rapid, i.e. spanning not less than three to four hours. Reduction of the temperature below $20^{\circ}\text{C}.$ rapidly diminished the capacity for recovery, although in some instances survivals occurred following depression to temperatures ranging from 17° to $10^{\circ}\text{C}.$ Below 17° , however, additional heating usually was required; in the rewarming process, for example, of eight animals lowered to $10^{\circ}\text{C}.$, four recovered with artificial heating, two recovered spontaneously, and two died despite the application of external heat.

The present report illustrates in a larger mammal the ability to withstand severe reductions in temperature, with recovery upon rapid rewarming and with no evident after-effects, and further illustrates the marked degree of bradycardia which can be tolerated. Moreover, loss of rigidity in the dog, under the conditions of the present experiments, is not the infallible index of irreversible cooling which it is reported to be in the human being (5), in which attempts at resuscitation, initiated during the flaccid state, were uniformly unsuccessful. In our experience, complete recovery was achieved in every instance from a stage of hypothermia considerably below the point at which muscle tone and reflex patterns were completely lost.

It seems probable that a severe hypoxia does not present a major problem to the hypothermic organism during the cooling process, in spite of an exaggerated A-V O_2 difference (5, 14) since under conditions of cooling one might reasonably anticipate a depression in the demand for oxygen. In favor of this is the observation that the survival time of the brain following interruption of its arterial supply is reported to be increased by cooling (W. Noell, cited by Alexander, 5). This circumstance, of course, may not hold true during the recovery period.

SUMMARY

The ability of normal dogs to withstand and to recover from marked cooling has been studied. Lightly anesthetized animals were submerged in iced water (2° to $9^{\circ}\text{C}.$) until a cardiac or respiratory crisis occurred. Resuscitation measures included rapid rewarming in water at 42° to $45^{\circ}\text{C}.$, and artificial respiration as required.

Thirteen animals survived a reduction in deep rectal temperature to an average of $14.9^{\circ}\text{C}.$ (range 11.7° to $16.8^{\circ}\text{C}.$). Recovery in each instance was complete. In two animals the rectal temperature was reduced to $15^{\circ}\text{C}.$ or below on two occasions. Shivering did not appear to play an important rôle in the rewarming process; it set in at widely variant stages of recovery and varied in degree from mild to intense.

In cooling, the fall in body temperature was linear, a characteristic which in

its early phase probably was influenced by the pre-immersion anesthetic. The visceral-rectal temperature gradient became more pronounced as cooling proceeded. Comparison is shown between rate of cooling of the living and the dead body.

Decline in body temperature was accompanied by a progressive fall in arterial blood pressure and by a bradycardia which became intensified as temperatures fell below 20°C. The slow heart rate was uninfluenced by vagotomy or by atropinization.

Eight animals failed to respond favorably, or expired before rewarming was begun. Death was clearly a matter of respiratory failure in some; in others, it was apparently attributable to heart block. In one preparation, in which the rectal temperature fell to 11.5°C., 'rewarming death' occurred at a rectal reading of 18.2°C.

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OBSERVATIONS ON ENERGY METABOLISM AND WATER BALANCE OF MEN SUBJECTED TO WARM AND COLD ENVIRONMENTS¹

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Received for publication December 30, 1947

The opportunity to obtain data on energy metabolism and water balance occurred during experiments designed to determine the influence of environmental temperature on blood volume of men (1). It seems worthwhile to publish these results as a supplement to the growing and large, but probably still inadequate, volume of available information on these subjects. In the field of energy metabolism, discussions center about whether the resting, fasting metabolism of men is greatly increased (in the absence of shivering) in the cold and decreased in the heat, and whether the relative proportion of calorogenic substances (carbohydrate, fat and protein) metabolized is in any way influenced by ambient temperatures. Some recent reports (2, 3) suggest that a small increase in human metabolism may be present after several days of exposure to cold. Such results are in accord with the delayed response to cold of animals (rats and rabbits) in which a large metabolic increase can be induced (4, 5), but the degree of metabolic stimulation reported for the human subjects seems to be considerably less than has been stated in earlier literature (see 6). The metabolic mixture preferentially selected and burned is popularly believed to be influenced by environmental temperature. The results of at least one laboratory experiment (7) support the belief that diets of higher fat content are chosen by men exposed to cold. On the other hand, it has been demonstrated recently that a high fat diet (consisting solely of pemmican) is not immediately acceptable to soldiers during maneuvers under subarctic conditions (8).

Various conclusions have been reached with regard to environmental influence on water balance. Some investigators have been unable to demonstrate any consistent change in water reserves (7, 9, 10) as a result of altering environmental temperature, while in one study (11) exposure to heat seemed to lessen the water reserves. However, the belief persists (9) that the quantity of stored water is generally greater in warm than in cold environments.

EXPERIMENTAL METHODS

Resting, fasting metabolism was determined by collecting and analyzing expired air of subjects who breathed outside air (Tissot method). Caloric intake was estimated from weights of the various articles of food, using charts of food composition (12) and the usually employed caloric values of the food elements.

An approximate method for determining water balance, based upon the pro-

¹ This investigation was supported by a Life Insurance Medical Research Fund Grant to Professor H. C. Bazett.

cedure described by Wiley and Newburgh (13), was employed. Total water intake was considered to be the sum of water drunk as such, water present in food and water arising from combustion. Water drunk as such was determined by weighing the water container of each individual at the beginning and end of the period. Water present in food was determined from weights of food using charts of food composition. Only an average estimate for metabolic water could be made. This was taken to be the quantity of water formed in the oxidation of the average daily diet. One gram of fat, carbohydrate and protein was assumed to produce respectively, 1.07, 0.60, and 0.41 grams of water.

Total water loss was considered to equal insensible water loss plus water of urine and feces. Water in urine was determined by subtracting the weight of solids from weight of urine. Weight of urinary solids was calculated from determinations of the specific gravity using Long's coefficient (14). Feces were assumed to contain 65 per cent water. Insensible water loss was obtained by subtracting from insensible weight loss the weight of carbon dioxide produced which is in excess of the weight of oxygen utilized. Differences in weights between carbon dioxide formed and oxygen utilized were calculated by multiplying average daily weight in grams of fat, carbohydrate and protein consumed by -0.06 , $+0.4$, and $+0.08$, respectively.

Changes in amount of body tissue are neglected in these calculations. Since we were interested in demonstrating relatively large and sudden changes in water balance, this is presumably an unimportant consideration in our study in which caloric intake was fairly constant and approximately equal to caloric expenditure.²

OUTLINE OF EXPERIMENT

A detailed outline of the first two experiments has already been published (1). Briefly, two subjects, dressed in shorts, undershirts, and sandals, were studied in a controlled temperature room where they stayed continuously for periods of 9 to 14 days. In the first (summer) experiment, the room was maintained uncomfortably warm (33°C . D. B., 28°C . W. B.) for a period of four days. This was followed by a six-day period of cold (21°C . D. B., 16°C . W. B.) and a final four-day period in which the original warm conditions were reproduced. In the second (winter) experiment a cold period of two days was followed by seven days in the heat. The temperature of the cold and warm periods in the second experiment corresponded closely with those of the first. Wind velocity was approximately 50 cm. per second in all cases. Subjects were fed a mixed diet in unrestricted quantities. They were allowed complete freedom in requesting favorite articles of food, though a nutritionally adequate diet was presented each day. In actual practice, the cook exerted considerable influence on food selection since subjects

² Wiley and Newburgh (13) overfed a normal human subject to the extent of 1600-1800 calories per day for a period of 15 days. Careful water balance studies showed a total gain of water during this period of only 897 grams though most of this was gained during the first few days. In comparison with this subject, over- or underfeeding of our subjects must have been of minor degree.

often did not express preference. With the clothing worn (shorts and undershirts) subjects were near the shivering point during exposure to cold and uncomfortably warm during exposure to heat.

In a third experiment, four subjects were studied during several exposures of a single day to heat or cold. Each period began at 8 A.M. with subjects in the resting, fasting state and was terminated at the same time on the following morning. The subjects were then free for a day or two before another experimental period was begun.

RESULTS AND DISCUSSION

There is little evidence of any marked change in energy metabolism in the summer experiment (table 1). Elevated values for resting, fasting metabolism were recorded occasionally (in both cold and warm environments) following a few days of exposure to cold. (In *subject Y* there was only one value during this period which was above the range of values recorded up to that point.) The two-day period of cold in the winter experiment did not result in any very obvious trend in subsequent values for basal metabolism. Failure to stimulate metabolism in any great degree by the rather severe degree of exposure to cold supports the results of some of the more recent studies (2, 3) over those reported earlier (see 6). The discrepancy between our observations on men, and those made on animals (4, 5) in which a large (10 to 30 per cent) increase in basal metabolism is produced by a previous exposure to cold, may be due to the fact that the animals were subjected to a more severe degree of exposure. There is a suggestion in the present study and other recent studies (2, 3) that cold may cause a delayed stimulation of metabolism of man, as it does in animals.

Daily caloric intake was for the most part quite constant in each experiment, and was not obviously influenced by the temperature of the environment.³ However, it does not necessarily follow that daily energy output remained unaffected by environment, for the subjects were not in exact metabolic balance. The composition of the diets during the warm periods of the summer experiment were: fat, 35-38 per cent; carbohydrate, 41-45 per cent; protein, 19-21 per cent. During the cold periods diets tended to be lower in fat (30-35 per cent) and higher in carbohydrate (46-50 per cent) with protein remaining at 19-21 per cent. Somewhat similar results were obtained in the winter experiment, the diets in the cold containing less fat and more carbohydrate and protein than the diets in the heat. The winter experiment is less satisfactory than the summer experiment for analysis, because of the scarcity of data during cold exposure. However, in each case the variation in proportion of dietary fat with exposure temperature seems to be correlated with the amount of milk consumed, i.e., milk was the article of diet which supplied the additional fat ingested in the heat. Milk con-

³ An abrupt change in caloric intake occurred in *subject R* toward the end of the summer experiment when his caloric intake fell by 15-20 per cent and remained at this lower level until the termination of the experiment. However, this change occurred during exposure to cold and continued throughout the ensuing period of heat; it was probably caused by an unhappy turn of events which occurred in the subject's personal affairs at about this time.

sumed during periods of heat (both summer and winter) varied between 465 and 1106 grams per day; during cold exposure, milk consumption varied between 232

TABLE 1. DATA ON ENERGY METABOLISM OBTAINED DURING THE SUMMER EXPERIMENT OF 1946

Body weights in kgm. (wt.) were obtained at 10 P.M. (after urinating) using scales sensitive to a few grams. Basal metabolisms in Cal/m²/hr. (BMR) and respiratory quotients (R.Q.) were determined at 8 A.M. by collecting expired air (Tissot). Total daily caloric intake expressed in units of 1000 large calories (Tot. Cal.) and daily caloric intake of fat (Cal. fat), carbohydrate (Cal. Cho.) and protein (Cal. Prot.) were estimated from weights of food using standard charts of food composition. Room temperature during the warm periods was 33°C., D. B., 28°C., W. B.; during the cold periods, 21°C., D. B., 16°C., W. B. During the afternoon of the last day of each period, room temperature was changed; only observations completed before the temperature was changed are recorded.

DATE	SUBJ.													
	R							Y						
	Wt.	BMR	R.Q.	Cal. fat.	Cal. cho.	Cal. prot.	Tot. cal.	Wt.	BMR	R.Q.	Cal. fat	Cal. cho.	Cal. prot.	Tot. cal.
A. Warm														
15	61.2							52.8						
16	61.2			0.95	1.31	0.58	2.84	52.7	37.6	0.79	0.70	1.27	0.58	2.55
17	61.2	39.0	0.81	1.04	1.08	0.43	2.55	52.6			0.91	0.83	0.37	2.11
18	61.8			0.87	1.16	0.53	2.56	52.7	39.4	0.80	0.77	0.91	0.49	2.17
19		38.7	0.85											
Mean.....	61.4	38.9	0.83	0.95	1.18	0.51	2.65	52.7	38.5	0.80	0.79	1.00	0.48	2.28
B. Cold														
20	61.2			0.97	1.10	0.55	2.62	52.4	38.1	0.80	0.67	0.83	0.48	1.98
21	60.5	39.7	0.84	1.03	1.15	0.40	2.58	51.9			0.62	0.95	0.37	1.94
22	61.1			1.02	1.36	0.51	2.89	52.3	39.1	0.87	0.75	1.19	0.39	2.33
23	61.3	41.1	0.83	0.91	1.37	0.56	2.84	52.3			0.65	1.07	0.47	2.19
24	60.8			0.69	1.09	0.43	2.21	52.4	36.8	0.85	0.56	1.24	0.48	2.28
25		42.2	0.84											
Mean.....	61.0	41.0	0.84	0.92	1.21	0.49	2.63	52.2	38.0	0.84	0.65	1.06	0.44	2.14
C. Warm														
26	61.2			0.79	0.84	0.34	1.97	53.0	42.6	0.86	0.91	0.86	0.31	2.08
27	61.2	40.6	0.80	0.93	0.82	0.49	2.24	53.9			1.07	1.08	0.51	2.66
28	60.9			0.74	1.09	0.46	2.29	53.7	38.4	0.85	0.55	0.81	0.57	1.93
29		39.4	0.83											
Mean.....	61.1	40.0	0.82	0.82	0.92	0.43	2.17	53.8	40.5	0.86	0.84	0.92	0.46	2.22

and 334 grams per day. This was in part due to the fact that subjects preferred a cool drink (milk) in the heat and a warm drink (hot tea) in the cold. The necessity for ingesting large quantities of fluid in the heat was probably also a

factor in determining the quantity of milk consumed. It is not believed that heat caused any specific 'appetite' for fat, and this is supported by the fact that butter consumption was, if any significant changes occurred, decreased in the heat. These results do not disprove the general belief, based upon dietary habits of natives living in cold regions⁴ and upon the results of other experiments (7), that diets high in fat are selected in cold environments. Since selection of diet at a given environmental temperature may be influenced by water contents of food available and by the temperature at which these foods are customarily

TABLE 2. DATA ON ENERGY METABOLISM OBTAINED DURING THE WINTER EXPERIMENT OF 1946-47

This table is prepared in the same manner as table 1. Room temperature during the warm period was 33°C., D. B., 28°C., W. B.; and 21°C., D. B., 15°C., W. B. during the cold period.

DATE	SUBJ.													
	R							Y						
	Wt.	BMR	R.Q.	Cal. fat	Cal. cho.	Cal. prot.	Tot. cal.	Wt.	BMR	R.Q.	Cal. fat	Cal. cho.	Cal. prot.	Tot. cal.
A. Cold														
26	59.5							51.3						
27	60.4			0.64	1.57	0.44	2.65	51.1	38.1	0.79	0.79	1.27	0.60	2.66
28		37.9	0.84											
Mean.....	60.0	37.9	0.84	0.64	1.57	0.44	2.65	51.2	38.1	0.79	0.79	1.27	0.60	2.66
B. Warm														
29	61.9			0.72	1.35	0.30	2.37	52.8	39.2	0.81	1.17	1.34	0.54	3.05
30	61.8	36.4	0.77	1.03	1.37	0.43	2.83	52.5			0.81	1.11	0.28	2.20
31	62.5			0.81	1.15	0.39	2.35	52.8	40.8	0.84	0.81	1.32	0.39	2.52
1-1	62.4	37.1	0.86	0.72	1.73	0.34	2.79	52.9			0.54	1.29	0.28	2.11
1-2	63.0			1.11	1.46	0.55	3.12	52.5	37.5	0.80	0.82	1.10	0.49	2.41
1-3	62.6	39.2	0.88	1.16	1.27	0.39	2.82	52.9			1.18	1.12	0.36	2.66
1-4									37.7	0.79				
Mean	62.4	37.6	0.84	0.93	1.39	0.40	2.71	52.7	38.8	0.81	0.89	1.21	0.39	2.49

served, it is not likely that human preferences for one or another of the calorogenic components of diet can be determined simply. A recent report on food selection of rats seems to demonstrate that carbohydrate is the variable element in dietary composition when caloric intake is altered by environmental temperature (15), but it cannot be assumed that these results on animals are applicable to man. Influence of environment on food selection is closely allied with the problem of altering the level of performance in environmental extremes by supplying diets of

⁴ It may be noted that Greenland Eskimos are said to eat very little fat or blubber as such (18); however, certain parts of animals that are consumed, e.g., liver, are rich in fat.

different composition. Recent studies on human subjects indicate that performance in the cold is bettered by diets high in fat or carbohydrate over performance when diets high in protein are fed (16, 17). It may be noted that a smaller consumption of food energy (162 per cent of basal requirements) and a

TABLE 3. DATA ON WATER BALANCE OBTAINED DURING THE WINTER EXPERIMENT OF 1946-47

Each period of water balance (B) covered the period from 10 P.M. one day to 10 P.M. the next. Water drunk (w) was determined by weighing water containers; water in food (f), from weights of food with the aid of charts of food composition; metabolic water (m), from daily food consumption (assuming metabolic balance). Water lost in urine (u) was determined from weights and specific gravities of urine, water in stools (s) was estimated from weights of stools; and insensible water losses (i) from insensible weight losses. All values are in grams. Room temperatures are given in table 2. On the days room temperature was changed, the first set of figures cover the period up to the time (5 P.M.) the temperature controls were adjusted, and the second set of figures cover the period from 5 P.M. to 10 P.M.

DATE	SUBJ.																		
	R									B	Y								
	Water intake				Water loss				Water intake				Water loss				B		
	w	f	m	Tot.	u	s	i	Tot.	w		f	m	Tot.	u	s	i		Tot.	
A. Cold																			
12-27	0	2268	348	2616	888	0	870	1758	+858	128	1827	320	2275	1875	56	565	2496	-221	
B. Transition from cold to warm																			
12-28	0	1245	275	1520	886	63	605	1554	-34	165	1115	253	1533	862	0	408	1270	+263	
12-28	271	685	73	1029	115	50	295	460	+569	935	518	67	1520	151	211	268	630	+890	
C. Warm																			
12-28	1719	1571	348	3638	665	0	1947	2612	+1026	1851	1954	320	4125	1155	96	2265	3516	+609	
12-30	651	1863	348	2862	682	247	1948	2877	-15	1812	1374	320	3506	1637	98	2033	3768	-262	
12-31	1537	1683	348	3568	753	0	2082	2835	+733	1619	1514	320	3453	715	96	2247	3058	+395	
1-1	1505	1630	348	3483	1594	95	1888	3577	-94	1424	1364	320	3108	1066	84	1802	2952	+156	
1-2	1655	1966	348	3969	903	366	2057	3326	+643	1536	1628	320	3484	1571	112	2129	3812	-328	
1-3	1011	1892	348	3258	1193	185	2111	3489	-231	1433	1650	320	3403	721	137	2149	3007	+396	
D. Transition from warm to cold																			
1-4	700	1084	275	2059	874	68	1759	2701	-642	736	921	253	1910	696	0	1817	2513	-603	
1-4	184	697	73	954	—	325	202	—	—	70	686	67	823	295	129	149	573	+278	

greater average weight loss (3.4 kgm.) occurred when diets high in protein were fed than occurred on feeding diets high in carbohydrate (191 per cent and 2.1 kgm. respectively). Relative undernourishment of subjects fed diets high in protein may have been partly responsible for differences in response to cold. A direct comparison between diets high in fat and diets high in protein was not made.

Both subjects gained weight in the winter experiment (table 2) and this was associated with approximately an equal gain in water as judged by water balance studies (table 3). The close agreement may be fortuitous for the daily water balance is inaccurate because of error introduced by the assumption of metabolic balance. Summation of these errors over a period of many days may indicate a change in a certain direction in water balance whereas in reality the opposite may have occurred. The daily water balances indicate that water was retained in large quantities during the first 29 hours of exposure to heat (from 5 P.M. on 12-28 to 10 P.M. on 12-29). Since the period from 5 to 10 P.M. on 12-28 is a period in which water normally seems to be stored (probably because of the evening meal), the significance of water retention during this time in our experiments may be doubtful. However there remains the fact that water retention of large magnitude occurred during the subsequent day. The quantities involved are too

TABLE 4. DATA ON WATER BALANCE OBTAINED IN SEVERAL EXPERIMENTS OF ONE-DAY DURATION (FROM 8 A.M. TO 8 A.M.)

Water balances were obtained in the manner illustrated in table 3. Room temperature was 33°C., D. B., 29°C., W. B. in the warm periods and 21°C., D. B., 18°C., W. B. in the cold.

DATE	SUBJ.							
	A		B		D		K	
	Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold
7/21	+674		-403					
7/24		-173		-207				
7/26	+261		-50		+1830			
7/31	+174		+408		+772		+224	
8/2		-292		-431		+362		-429
8/5	+513		-279		+549		-1	
Means.....	+406	-233	-162	-319	+788	+362	+112	-429

great to result from formation of new tissue; and presumably the water was stored in extracellular spaces. A causal relationship between warming the environment and increasing body water content is, of course, not established by this single study. Furthermore, the experiment was not suitably designed to demonstrate water loss on exposure to cold. In this particular experiment, the initial exposure to cold was complicated by a change in the general living condition of the subjects, i.e., subjects were not only exposed to a temperature change during the single day of cold for which data for water balance are available, but they also began living in the controlled temperature room. In the final cold period, data on water balance for only a fraction of a day were obtained.

In the summer experiment body weights of both subjects decreased on exposure to cold and increased on exposure to heat. In one subject (*R*) transfer from cold to heat took place very soon after caloric intake of this subject dropped sharply, but even in this case average body weight during exposure to cold was less than

during either period of heat exposure. These changes in weight, for the most part, are presumably too large and abrupt to be caused by caloric imbalance, i.e., formation or destruction of body tissue. The data on water balance in this experiment are not presented since records were not kept with sufficient accuracy to be reliable.

The water balance studies made during exposure for only one day to heat or cold (table 4) are also complicated by the change from home life to living in the controlled temperature room. On the average, however, the influence of environmental temperature was apparent in each subject. Two subjects (*A* and *K*) exhibited a positive water balance during exposure to heat and a negative balance in the cold. Subject *D* gained water under both conditions but gained more in the heat and the remaining subject (*B*) lost water both in the heat and cold but on the average lost more in the cold.

All considered, our results are fairly consistent in showing an effect of environmental temperature on water storage. Burton *et al.* (7) obtained equivocal evidence of similar changes in their experiments which were thought to be complicated by changes in amount of body tissue. Conley and Nickerson (10) found in two of four experiments a decrease in thiocyanate space on transferring subjects from heat to cold; in the other two experiments, and in two additional experiments in which subjects were transferred from cold to heat, a significant change in this fluid compartment was not found. Their error of measurement was estimated at three per cent, which would amount to about one-half liter in an individual of average size. Forbes *et al.* (11) found an average decrease in interstitial fluid of 11 per cent in a group of ten individuals transferred from Massachusetts to Mississippi. However, the variation among individuals was from -34 to $+26$ per cent. Apparently extraneous factors must have been present to account for such large fluctuations in water reserves.

SUMMARY

1. Studies of energy metabolism and water balance were made on two subjects living in a controlled temperature room. In one experiment, a warm period ($33^{\circ}\text{C}.$, D. B., $28^{\circ}\text{C}.$, W. B.) of four days was followed by six days of cold ($21^{\circ}\text{C}.$, D. B., $16^{\circ}\text{C}.$, W. B.) and a terminal period of four days of heat. A second experiment consisted of two days of cold followed by seven days of heat and a final brief period of cold. Four other subjects were studied in a series of experiments consisting of exposure for a single day to either heat or cold.

2. Energy metabolism was not affected greatly by these environmental extremes as judged by records of caloric intake, dietary composition, basal metabolism and resting, fasting respiratory quotients. Values for basal metabolism in one experiment were on the average slightly higher following exposure to cold for a few days than they were prior to exposure, but the differences are small. Diets consumed in the heat contained somewhat more fat and less carbohydrate or protein than diets consumed in the cold. The increased milk consumption, which was responsible for the additional dietary fat in the heat, did not seem to be the result of any specific 'appetite' or 'desire' for fat.

3. Evidence for storage of water on changing from a cold to a warm environment and loss of water under opposite circumstances was obtained from water balance studies and observations of body weight. The quantities of water involved ranged from a few hundred cubic centimeters to more than a liter.

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REACTIONS OF MEN EXPOSED TO COLD AND WIND

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Received for publication September 3, 1946

The contribution made by outer garments of low air permeability to the overall insulative value of cold-weather garments and to the thermal balance and comfort of the wearer is uncertain, although no one doubts either the value or the necessity of an outer windproof layer. Since the increased heat losses which occur with wind arise primarily from air exchange within the clothing, the thicker and the more permeable the under garments, the greater is the necessity for a good windproof. It has been suggested that the degree of windproofing required depends upon the relative permeability of the underlying layers. If the layer most immediate to the windproof outer garment offers some resistance to air movement, the increased heat loss by convection and air exchange with wind is consequently diminished. This can be illustrated by comparing thermal experiences under the two conditions of use of the alpaca-mohair pile garment of the Arctic issue. If the pile is placed outside, next to the windproof garment, greater heat losses occur with wind than when the backing of the pile is worn adjacent to the outer garment.

Troops, especially mechanized units, operating in regions of extreme cold are faced with the problem of both cold and wind. Siple's (1) data on wind chill and the reports of Horvath *et al.* (2) on men exposed to cold without wind could not be extended to these conditions. Since the data available were inadequate to determine either the degree of stress or the type of response that would occur in men exposed to cold and air movement, studies were conducted on soldiers seated quietly in environments where both temperature and wind velocity were controlled.

METHODS

These experiments were conducted in the laboratory cold room for the most part at an environmental temperature of -23°C . Other temperatures employed were -17° and -36°C . Previous experience with the Arctic issue had demonstrated that at -17°C . without air movement men could sit quietly in relative comfort for periods of four to five hours. However, exposure at an ambient temperature of -17°C . would necessitate rather long experimental periods and other factors such as hunger, boredom, etc. might become deciding elements. It was also known that the Arctic issue offered adequate protection for two to three hours at -29°C . Therefore, it was felt that an intermediate temperature of -23°C . would enable differences to be detected without any of the disadvantages of the lower or higher temperatures.

¹ This data was obtained while the author was at the Armored Medical Research Laboratory, Fort Knox, Ky.

A tunnel 18 feet long, 7 feet high, and 5 feet wide was constructed in the cold room. Six fans were so placed that when air was drawn through the tunnel turbulence was minimal. The tests were conducted at three wind velocities: 0, 5, and 9.6 mph. By placing two subjects on tables, four men could be exposed simultaneously to an uninterrupted air stream. In all of the tests a copper cylinder, wrapped in the four layers of the complete Arctic issue, was suspended from the roof of the tunnel in the midst of the subjects, and the rate of cooling of the contained water was measured.² This physical method, employed as a measure of environmental stress, indicated that constant conditions were maintained during the experiments.

Ten representative subjects were selected from a group of 50 men whose subjective responses to cold had been previously determined. Originally, of the five subjects selected for detailed study, three were classified as resistant and two as susceptible to cold. Unfortunately, considerable difficulty was experienced in outfitting the men, so that four men who had been tentatively classified susceptible and only one resistant were finally employed. During the progress of the test, however, it became evident that this classification of relative resistance was uncertain and was subject in the majority of cases to considerable daily variation, so that the failure to have equality at the outset in number of 'resistants' and 'susceptibles' was not very important.

A standardized procedure was followed prior to each day's test. The men arrived at 0645 hours, ate a standard breakfast, and then rested until they dressed for the test which began at 0830 hours. Tests were conducted both morning and afternoon but only those data obtained during the morning experiments are presented here, since the subjects' reactions differed somewhat from morning to afternoon.

Skin temperatures were obtained on chest, upper arm, thigh, calf, and dorsal surface of the foot of each of the four subjects in the tunnel. In some cases ten copper constantan thermocouples were worn. Continuous measurements of oxygen consumption were made on two of the subjects, one with an open-circuit and the other with a closed-circuit apparatus. Rectal temperatures were obtained by clinical thermometers. The clothing worn by the subjects is listed below.³ A complete set of tests on all subjects in each of the windbreaks was impossible. Nevertheless, a sufficient number of tests was made on all three of these clothing items, with at least two subjects wearing each of the items, so that general conclusions may be drawn.

² This device was suggested by Dr. Theodore Hatch.

³ Standard Arctic clothing worn by subjects consisted of the following: drawers, wool, 50/50; undershirts, wool, 50/50; shirt, flannel, O.D.; trousers, field, wool, O.D.; trousers, field, pile; trousers, field, cotton, O.D. (sateen, 9 oz.) windproof; parka, field, cotton, O.D. (sateen, 9 oz.) windproof; parka, pile; shoe, Arctic, felt; socks, wool, ski (2 pairs); socks, wool, cushion sole (1 pair); mittens, insert, trigger finger M-1943; mittens, shell, trigger finger M-1943; mufflers, wool; and wristlets, knit.

Three types of windbreak materials for the outer garments were used: a) nylon-air permeability of 0 cu. ft./sq. ft./min.; b) sateen, 9 oz.—air permeability of 5 cu. ft./sq. ft./min. (this is identical to the material used in the control parka and trousers); and c) herringbone twill-air permeability of 20 cu. ft./sq. ft./min.

RESULTS AND DISCUSSION

Effect of wind upon insulation. Data on the insulative value of the standard Arctic clothing, the sateen outer garments having an air permeability of 5 cu. ft/sq. ft/min., are presented in table 1. Hourly Clo values, determined by the standard procedure (3, 4) are presented in the table together with the average Clo values for the second and third hours. The Clo values as determined from the third-hour metabolic rate and the predicted equilibrium temperature, θ_e , are also

TABLE 1. INSULATIVE VALUE OF THE STANDARD ARCTIC CLOTHING IN RELATION TO WIND VELOCITY

SUBJECT	WIND VELOCITY	INSULATION				
		Clo Units, by standard method				Clo Units by θ_e
		First hour	Second hour	Third hour	Average of 2nd and 3rd hours	
	<i>mph.</i>					
GO	0	3.1	3.4	—	3.4	5.2
	5	4.3	3.9	5.5	4.7	5.0
	9.6	2.7	3.1	4.1	3.6	4.0
HE	0	3.5	5.7	3.8	4.7	5.3
	5	2.9	4.3	3.2	3.8	4.5
	9.6	2.7	3.8	2.8	3.3	3.7
GR	0	4.1	4.3	5.1	4.7	4.9
	5	3.6	4.2	4.4	4.3	4.2
	9.6	3.3	3.5	2.8	3.2	3.7
VA	0	3.8	5.0	4.8	4.9	5.0
	5	3.2	5.0	3.8	4.4	5.2
	9.6	3.5	4.5	4.3	4.4	5.5
BA	0	2.5	4.3	3.0	3.7	3.9
	5	2.3	4.8	4.2	4.5	4.1
	9.6	5.2	2.9	3.7	3.3	4.1
Average for all subjects.....	0	3.5	4.5	4.2	4.4	4.9
	5	3.4	4.4	4.2	4.3	4.6
	9.6	3.5	3.5	3.5	3.5	4.2

given for the five subjects (5). All values are corrected for the insulation of the surrounding air. During the three-hour exposure, the apparent insulation of the Arctic issue was changing; it appeared to increase with time under still-air conditions. This was true to a lesser extent at 5 mph. wind. But with an air movement of 9.6 mph., there was, after the first hour, a fairly consistent decrease in insulative value with increasing wind velocity.

No material differences in the insulation afforded by the Arctic clothing modified by substitution of outer garments having different air permeabilities were noted at zero wind velocities (table 2). When the wind velocity was increased to

5.0 and 9.6 mph., the Clo values decreased in all cases except when the nylon outer garment was worn. The high average insulation at 9.6 mph. in this case, however, resulted from an extreme value, 7.0, on one of the two subjects, while in

TABLE 2. INSULATIVE VALUE OF STANDARD ARCTIC CLOTHING IN RELATION TO THE AIR PERMEABILITY OF THE OUTER GARMENT LAYER
(Average values from two to six subjects in Clo units, calculated from θ_c)

ARCTIC CLOTHING WITH OUTER GARMENTS OF THE FOLLOWING WINDPROOF MATERIALS	WIND VELOCITY, MILES PER HOUR		
	0	5.0	9.6
Nylon ¹	5.3	4.3	5.8
Sateen—9 oz.....	4.9	4.6	4.2
Herringbone twill.....	5.4	4.3	3.6

¹ Two subjects.

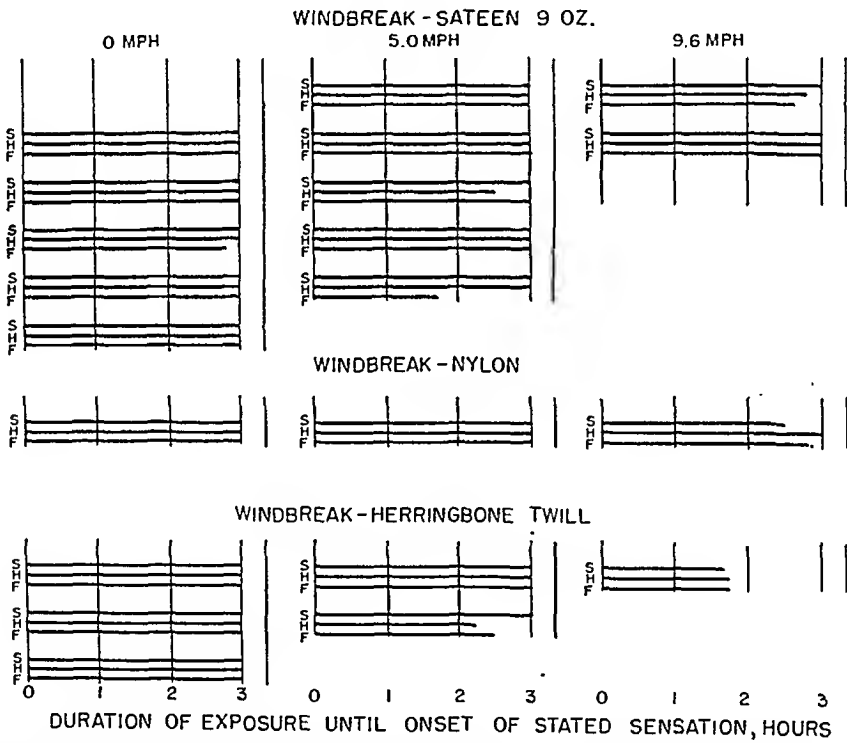


FIG. 1. SUBJECTIVE REACTIONS of *Subject HE* exposed at $-23^{\circ}\text{C}.$ to varying wind velocities while dressed in Arctic issue and different windbreaks. F = onset of pain in feet; H = onset of pain in hands; S = onset of shivering. Extension of line to 3 hrs. indicates no stated sensation during exposure.

the other the insulation value was lower than at either 5.0 or 0 miles per hour. The greatest loss of insulation with wind occurred with the herringbone twill wind-proof, the average Clo value dropping from 5.3 to 3.6.

The decrease in the apparent insulative value of the clothing assembly with increasing air movement no doubt resulted from interference with the stability of

the bound air layer. Insulation of clothing is essentially determined by the thickness and stability of the air immobilized between fabric layers and within the fabrics themselves. There is a possible three-fold variation in the insulation from a completely static air layer to one within which there is free convection. At high-wind velocities two factors may be operating to alter the insulation: circulation within the clothing and internal circulation plus air exchange with the outside. Both may result in real increases in heat transfer.

Effect of wind on body-cooling rate. The advantage of windproof garments is demonstrated by the mean skin temperature curves in figure 4. This subject VA, was exposed to $-17^{\circ}\text{C}.$ for a period of five hours with and without air movement. The slightly greater decrease in the mean skin temperature at the 5 mph. wind

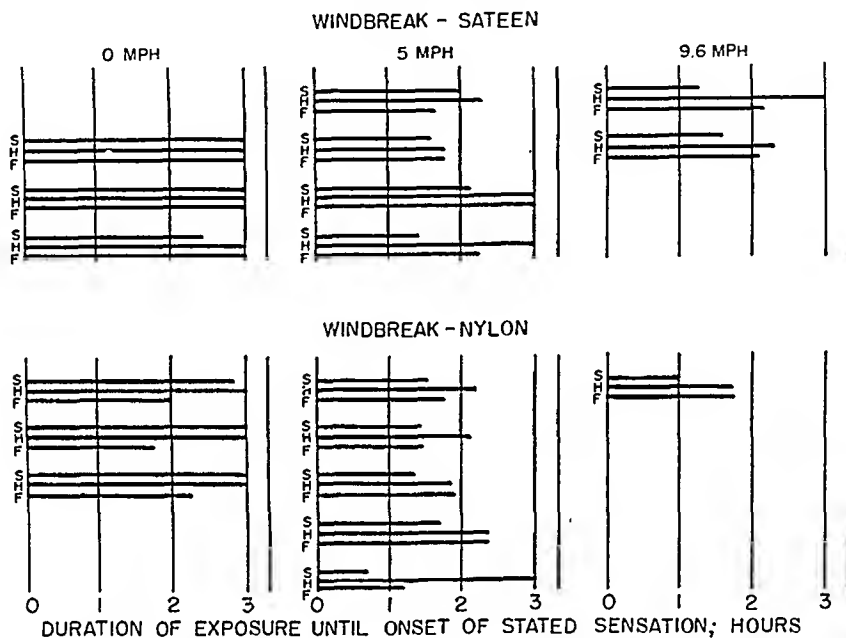


FIG. 2. SUBJECTIVE REACTIONS of *Subject GR* exposed at $-23^{\circ}\text{C}.$ to varying wind velocities while dressed in Arctic issue clothing modified by two different types of windproof garments. For explanation see fig. 1.

was evident from the very beginning. This initial rapid fall was stabilized quickly during the remainder of the experiment. In one of his repeat exposures to wind and cold, VA inadvertently made an 18-inch linear tear in the front of his parka. Since such events commonly occur, it was decided to continue the experiment. The mean skin temperature fell rapidly and continued to levels more commonly observed at much colder ambient temperatures. The subject complained very early about cold, discomfort and shivering. He was unable to continue for more than four hours.

The data on mean skin and rectal temperatures are summarized in table 4. Neither the values of the cooling constant, K , the mean skin temperature at the end of the exposure period nor the predicted equilibrium temperature, θ_e , exhibited great change with the type of outer garment being worn by these

men. However, slight trends in the average data relative to wind velocity were observed. The median values for the last mean skin temperature recorded were 26.0° , 25.2° and 24.8°C. at wind velocities of 0.0, 5.0 and 9.6 mph., respectively.

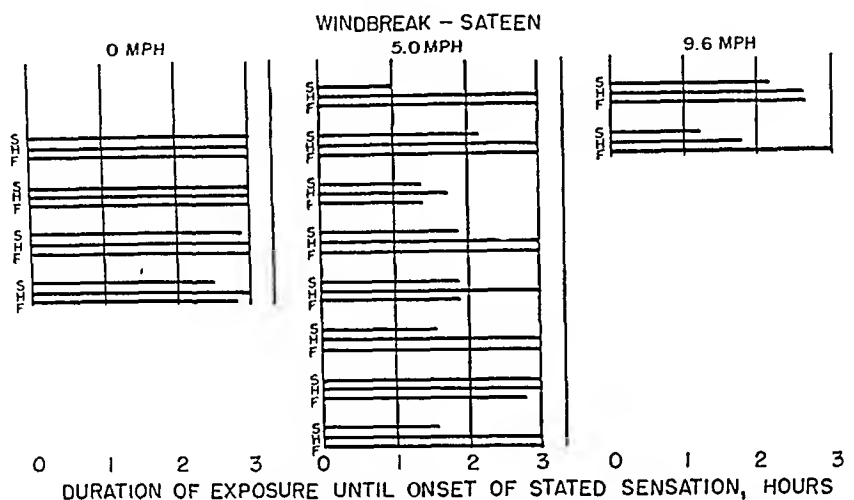


FIG. 3. SUBJECTIVE REACTIONS of *Subject VA* exposed at -23°C. to varying wind velocities. For explanation see fig. 1.

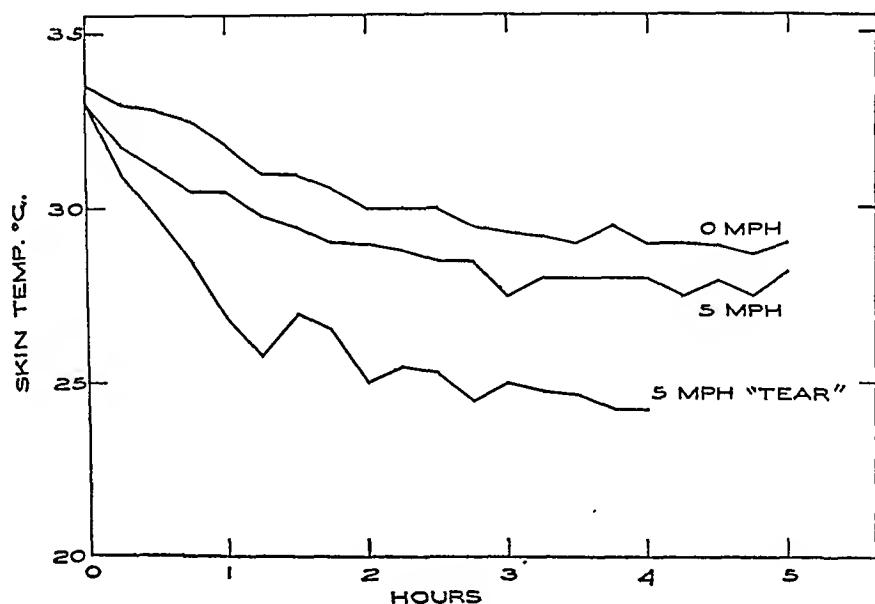


FIG. 4. MEAN SKIN TEMPERATURES of *Subject VA* exposed to -17°C. , illustrating the influence of wind and loss of protection as a consequence of an 18-inch tear in the windproof parka.

tively. The predicted equilibrium mean skin temperatures followed this same pattern but were approximately 1.2°C. lower. The median values for K , the cooling constant, were 0.720, 0.755 and 0.835 for the three wind velocities. These differences are not significant.

The fall in rectal temperature was not appreciably affected by either the ambient temperature or the wind velocity. The principal effect of these environmental conditions was reflected in the changes in mean skin temperatures. Although it appeared possible to generalize from these values and obtain information regarding equivalent environments such as have been developed for hot climates by Robinson *et al.* (6), it was felt best not to do so in view of the present state of our knowledge regarding exposure of men to cold.

The alterations in metabolic rate with low ambient temperature, air movement and the relative permeability of the outer garments were significant. Table 3

TABLE 3. HOURLY METABOLIC RATES¹ IN RELATION TO WIND VELOCITY STANDARD ARCTIC CLOTHING
(Averages for five subjects)

	0 MPH		5.0 MPH		9.6 MPH	
	Cals/M ² /hr.	Per cent increase	Cals/M ² /hr.	Per cent increase	Cals/M ² /hr.	Per cent increase
First hour.....	38.3		39.0		39.4	
Second hour.....	42.7	11	45.2	16	63.6	61
Third hour.....	51.3	34	60.1	54	67.8	73

¹ Corrected for heat lost in warming inspired air and water evaporation from lungs.

TABLE 4. MEAN SKIN AND RECTAL TEMPERATURES OBTAINED DURING THE SITTING EXPOSURES TO VARIOUS ENVIRONMENTS AND WIND VELOCITIES

ENVIR. TEMP.	WIND VELOCITY	DURATION OF EXPOSURE	INITIAL SKIN TEMP.	FINAL SKIN TEMP.	Δ SKIN TEMP.	INITIAL RECTAL TEMP.	FINAL RECTAL TEMP.	Δ RECTAL TEMP.
°C.	mph.	hours	°C.	°C.	°C.	°C.	°C.	°C.
-23	0.0	3	33.0	25.0	-7.0	37.3	36.8	-0.5
-23	5.0	3	33.1	25.2	-7.9	37.4	36.8	-0.6
-23	9.6	3	32.4	24.8	-7.6	37.3	36.7	-0.6
-17	0.0	5	33.1	27.3	-5.8	37.3	36.7	-0.6
-17	5.0	5	32.6	25.0	-7.6	37.3	36.6	-0.6
-36	0.0	3	33.3	24.7	-8.6	37.4	36.9	-0.5

presents such data for the standard Arctic assembly with the total caloric output corrected for heat lost both in warming the inspired air and by evaporation of water from the lungs. The metabolic response of the subject under these experimental conditions was approximately the same for the first hour at all wind velocities. During the second and third hours, however, a marked stimulus for extra heat production was evident. The higher the wind velocity, the sooner was the stimulus to extra heat production developed and the greater the response. In this connection, it is noteworthy that, as a rule, the greatest changes in stored heat occurred during the first hour. Variable but generally much smaller demands on this heat supply were made during the next two hours. Neither the absolute amount nor the rate of the heat loss from storage appear to correlate with the stimulus to extra heat production.

Men dressed in the nylon or sateen windproof garments had similar metabolic responses to increased air movement and duration of exposure. The herringbone twill garments offered less protection against wind, however, and the extra heat production of subjects dressed in these was greater than with the others, the hourly metabolic rates being 42, 93 and 85 Cal/M²/hr.

Subjective responses. The subjective responses of the men were significantly altered when they were exposed to cold and wind (fig. 1, 2, and 3). *Subject HE*, figure 1, was the most resistant subject in this study. When he wore adequate outer windproof garments (sateen or nylon), *HE* did not become uncomfortable even at the highest wind velocity. In herringbone twill outers, however, he failed to maintain comfort at 5.0 mph., and at a wind velocity of 9.6 mph., he was definitely uncomfortable. For the less resistant subjects, the herringbone twill was equally inadequate at 5.0 mph. wind. In figure 2 are recorded the subjective sensations for *Subject GR* in the two best windproof outer garments, sateen and nylon. He remained relatively comfortable at zero wind velocity, but with air movement his subjective sense of discomfort increased. *GR* appeared to be less comfortable when wearing the nylon than with the sateen outer garment. This is in striking contrast to the subjective experiences of *Subject HE* as shown in figure 1.

Exposure of men to rapid air movements diminishes the time from initial exposure to the appearance of sensations of discomfort. Although general body comfort, as evidenced by onset of shivering, appears to be most readily influenced by wind, the onset of discomfort in the extremities does not lag very far behind. Shivering, continued for any length of time, is a distressing experience, fatiguing and distracting. Fine muscular control is almost impossible and concentration is difficult, one thought dominating—the desire for termination of shivering. Under such conditions, the general overall efficiency is impaired and the necessity for adequate protection is quite clear.

The author wishes to express his appreciation to the men who so willingly subjected themselves to the tests and to Mr. James Gregg, Sgt. H. Golden, my wife, Elizabeth, and Mrs. James M. Nelson for their technical assistance.

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EFFECTS OF ACUTE EXPOSURE TO SIMULATED ALTITUDES ON DEXTROSE TOLERANCE AND INSULIN TOLERANCE

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Received for publication November 3, 1947

Although there have been numerous investigations of the effects of anoxia on fasting blood glucose levels, there is little data in the literature with regard to the effects of altitude on dextrose tolerance and insulin tolerance. Most investigators have reported that anoxia causes hyperglycemia (1, 5, 6, 7, 11, 13, 14, 15, 17, 18, 19) although some have reported that anoxia has no effect on fasting blood sugar (8, 10), and some have reported that even hypoglycemia may result under certain conditions from anoxia (16, 21). Middlesworth, Kline and Britton (20) have recently demonstrated that blood sugar levels may either rise, fall or remain unchanged under anoxia, depending upon the absorptive condition of the animal, length and degree of anoxic exposure and degree of acclimatization.

There are also contradictory reports in the literature regarding the effects of altitude on dextrose tolerance, which was found to be decreased (2), increased (8), and unchanged (3, 4, 16). . . D'Angelo (4) found a difference in the hypoglycemic component of the glucose tolerance curves between altitude and ground level, with the depression of blood sugar values below the pre-ingestion value being of greater magnitude and duration at ground level. This difference, however, could not be correlated with any difference in severity or duration of hypoglycemic attack. Reports on the effect of insulin on blood sugar levels under conditions of anoxia (10, 18, 19) are quite divergent as regards the incidence, type and severity of insulin convulsions observed, but they are in agreement on the fact that blood sugar levels obtained are no lower than those following similar doses of insulin at atmospheric pressure. We have found no reports of dextrose tolerance tests performed on unacclimatized animals immediately upon acute exposures to altitude anoxia, nor of standard insulin tolerance tests made under similar conditions. In the present study we have performed these two types of experiments.

METHODS

The animals used in these experiments were three well-trained male Dalmatian coach hounds, which were litter mates of a carefully inbred, pedigreed strain.

The dogs were loosely restrained in a supine position on animal boards. No anesthesia was used and all blood samples were obtained by external jugular puncture. The dogs were fasted for twelve hours prior to the tests.

In all experiments conducted under reduced atmospheric pressure, a standard procedure was adhered to under which the dog was restrained on the animal

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board in the altitude chamber and ascent to the desired altitude was accomplished at the rate of 2000 feet per minute. Within five minutes after arrival at the desired altitude the fasting blood sample was drawn. In order to minimize the acclimatization factor, an interval of at least one week was maintained between successive exposures of any dog to altitude.

For the intravenous dextrose tolerance tests, the test dose of dextrose used was 0.5 gram per kilogram of body weight. Blood samples were drawn immediately before and 30 minutes, one hour, 2 hours and 3 hours after the injection of the test dose.

The insulin tolerance test was done as described by Fraser, Albright and Smith (9) with the exception that .2 unit of insulin and .02 cc. of 1:1000 adrenalin per kilogram of body weight were used. Blood samples were drawn immediately before and 20 minutes, 30 minutes, 45 minutes, one hour, one hour and 30 minutes, and 2 hours after the intravenous injection of insulin. Immediately after the two-hour sample was drawn, the calculated amount of adrenalin was injected intramuscularly. Two more blood samples were then drawn at 45 and 60 minutes after this injection.

All blood sugar determinations were done by the Horvath and Knehr (12) method.

RESULTS

The results obtained with the dextrose tolerance tests at ground level², 18,000 feet and 24,000 feet are shown in table 1 and figure 1. Table 1 gives both the absolute blood sugar levels and the absolute changes from the initial (fasting) levels. The curves shown in figure 1 are plotted on the basis of the absolute changes in blood glucose level as compared to the initial (fasting) level, taken as zero.

It will be noted from these data that there are definite alterations in form of the dextrose tolerance curves at altitudes of 18,000 and 24,000 feet, respectively, as compared to the baseline values done at ground level². Although these alterations are not strictly parallel quantitatively from dog to dog, they are quite similar qualitatively as regards the general trends of the curves. For example, it is obvious from observation of these curves that all of the curves of the individual animals as well as the mean curve for the group show: 1) at 18,000 feet a 30-minute value approximately one half as high as the corresponding value at ground level, but at 24,000 feet a 30-minute value approximately of the same magnitude as the corresponding value at ground level; 2) at 18,000 feet a slower rate of fall of blood glucose level, so that in spite of the low 30-minute values, the one-hour levels are in every case higher than the one-hour values at ground level and at 24,000 feet an even slower rate of fall during the corresponding period; (in one case a marked rise in the blood sugar level appeared at one hour as compared to the 30-minute value) and 3) a failure of the glucose level to return to initial levels, either at 18,000 feet or at 24,000 feet in the three-hour period.

² Ground level as used here means 'ground level at Randolph Field, Texas, (approximately 750 feet)'.

Changes in insulin tolerance were not as marked as those in dextrose tolerance. Table 2 and figure 2 show the results of the insulin tolerance tests. It will be noted that the minimum levels of blood glucose obtained at altitudes of 18,000 feet and 24,000 feet were approximately the same and were not as low as those obtained at ground level. Furthermore, the response to adrenalin in *dogs no. 1* and *no. 2* was decidedly less marked at altitudes of 18,000 feet and 24,000 feet than at ground level.

It is interesting to note that the fasting blood sugar values for blood taken approximately five minutes after arrival at altitudes of 18,000 feet and 24,000 feet, respectively, are altogether comparable with the fasting values obtained at ground level.

TABLE 1. DEXTROSE TOLERANCE TESTS

ALTITUDE	TIME IN HRS.	BLOOD GLUCOSE LEVEL (MGS. PER 100 CC.)			BLOOD GLUCOSE LEVEL (EXPRESSED AS ABSOLUTE CHANGE FROM INITIAL FASTING LEVEL)			
		Dog 1	Dog 2	Dog 3	Dog 1	Dog 2	Dog 3	Average
Ground level	0	106	120	132	0	0	0	0
	$\frac{1}{2}$	190	212	199	84	92	67	81
	1	130	135	111	24	15	-21	6
	2	112	123	128	6	3	-4	2
	3	114	128	142	8	8	10	9
18,000 ft.	0	125	125	122	0	0	0	0
	$\frac{1}{2}$	171	168	154	46	43	32	40
	1	168	143	125	43	18	3	21
	2	131	145	143	6	20	21	16
	3	137	144	134	12	19	12	14
24,000 ft.	0	139	125	122	0	0	0	0
	$\frac{1}{2}$	227	199	177	88	74	55	72
	1	240	160	176	101	35	54	63
	2	282	155	171	143	30	51	75
	3	201	149	192	62	24	70	52

DISCUSSION

Although the data presented demonstrate considerable variation from animal to animal in response to altitude anoxia, certain trends are quite striking. These are 1) a decreased hyperglycemia responsiveness, more marked at 24,000 feet than at 18,000 feet, 2) an increased insulin resistance, 3) a decreased response to adrenalin and 4) a uniformly low 30-minute blood sugar level at 18,000 feet. This latter finding is difficult to explain since it would indicate an increased hyperglycemia responsiveness during the first 30 minutes following injection of dextrose in spite of a decreased hyperglycemia responsiveness in the remainder of the period studied.

It is interesting to note that the fasting blood sugar values obtained approximately five minutes after arrival at simulated altitudes of 18,000 feet and 24,000

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feet, respectively, are altogether comparable with the fasting levels obtained in these same animals at ground level. This strongly suggests that at this point of exposure to reduced oxygen tension the sympathico-adrenal activity is not

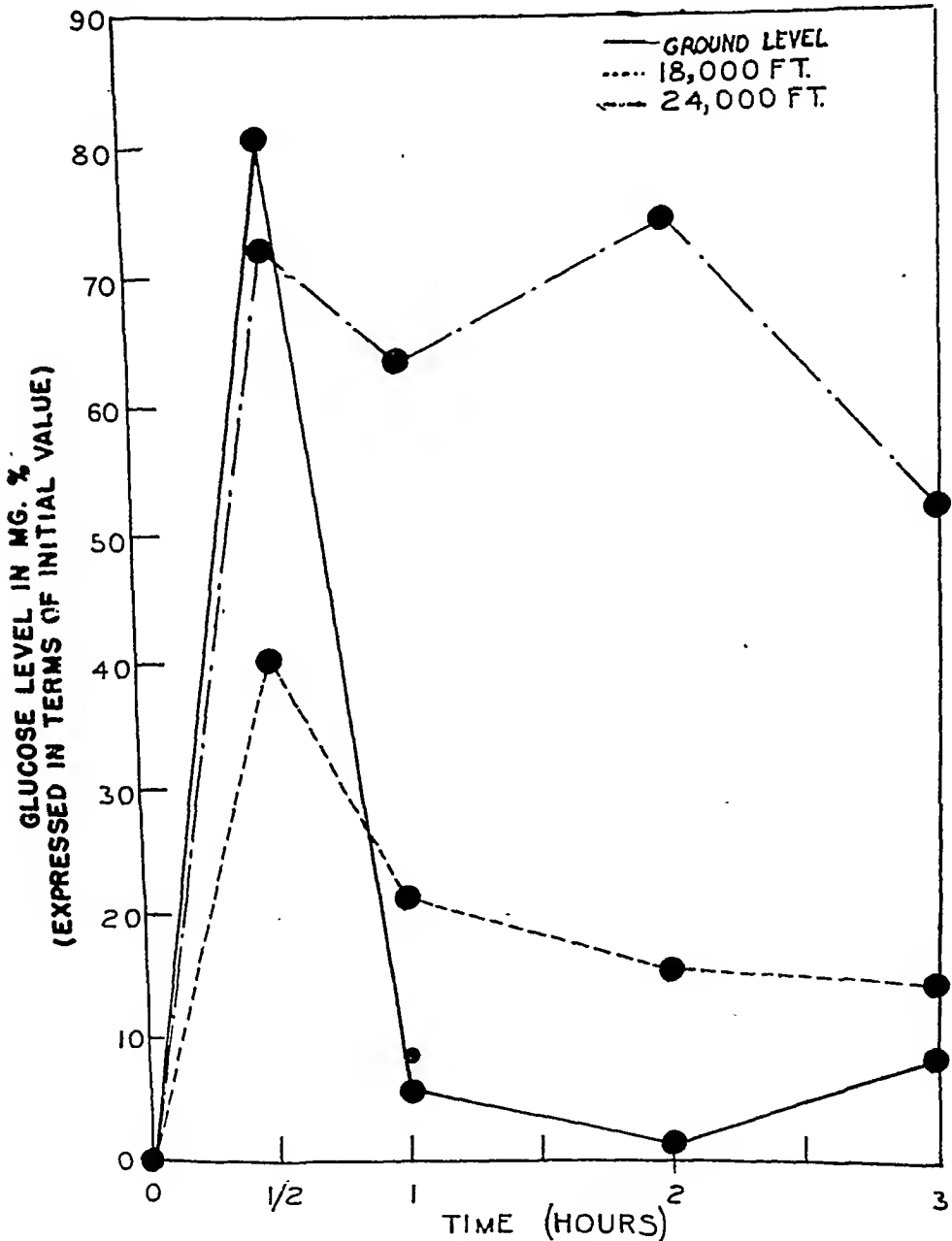


FIG. 1. MEAN DEXTROSE TOLERANCE CURVES on dogs 1, 2 and 3.

demonstrably increased, and one must therefore be cautious in invoking this mechanism to account for elevated blood sugar frequently noted under anoxic conditions.

It is not surprising that the data presented in this paper differ somewhat from

observations previously reported (2, 8, 16, 3) since none of these other experiments was performed under strictly comparable conditions. Acclimatization to altitude or prolonged exposure previous to the tolerance studies might well lead to marked variations in response to these tests. In view of the observations of Middlesworth, Kline and Britton (20) with respect to blood sugar levels rising,

TABLE 2. INSULIN TOLERANCE TESTS

ALTITUDE	TIME	BLOOD GLUCOSE LEVEL (MGS. PER 100 CC.)			BLOOD GLUCOSE LEVEL (EXPRESSED AS ABSOLUTE CHANGE FROM INITIAL FASTING LEVEL,			
		Dog 1	Dog 2	Dog 3	Dog 1	Dog 2	Dog 3	Average
Ground level	0	126	106	113	0	0	0	0
	20 min.	92	71	76	-34	-35	-37	-35
	30 min.	97	61	90	-29	-45	-23	-32
	45 min.	84	80	88	-42	-26	-25	-31
	1 hr.	108	87	133	-18	-19	+21	-5
	1½ hrs.	126	82	131	0	-24	+19	-2
	2 hrs.	142	99	131	+16	-7	+19	+9
	45 min.	149	122	136	+23	+16	+24	+21
	60 min.	156	125	—	+30	+19	—	+25
18,000 ft.	0	125	112	126	0	0	0	0
	20 min.	105	87	106	-20	-25	-20	-22
	30 min.	104	97	118	-21	-15	-8	-15
	45 min.	100	90	106	-25	-22	-20	-22
	1 hr.	136	117	108	+11	+5	-18	-1
	1½ hrs.	123	117	122	-2	+5	-4	0
	2 hrs.	123	114	122	-2	+2	-4	-1
	45 min.	138	120	126	+13	+8	0	+7
	60 min.	127	111	122	+2	-1	-4	-1
24,000 ft.	0	134	102	96	0	0	0	0
	20 min.	98	76	84	-36	-26	-12	-25
	30 min.	138	86	77	+4	-16	-19	-10
	45 min.	132	92	95	-2	-10	-1	-4
	1 hr.	132	112	—	-2	+10	—	+4
	1½ hrs.	127	116	111	-7	+14	+15	+7
	2 hrs.	171	126	105	+37	+24	+9	+23
	45 min.	143	128	112	+9	+26	+16	+17
	60 min.	158	120	120	+24	+18	+32	+25

falling or remaining unchanged under anoxic conditions depending upon absorptive condition of the animal, length and degree of anoxic exposure and degree of acclimatization, it would seem logical to assume that these factors would be important in determining the response to glucose and insulin tolerance tests.

It is not the purpose of this study to assign the alterations observed in these tests to any single metabolic or hormonal factor. Several factors may be of importance in bringing about these changes. Although the relative importance of the rôles played by these various factors cannot be stated at the present time,

the following must be considered as possible etiologic factors: 1) insufficient oxygen available to supply the energy necessary for the proper functioning of the enzymatic systems involved in carbohydrate metabolism; 2) increased activity of the sympathico-adrenal system; and 3) increased adrenal cortical activity. The relative importance of these various mechanisms is under investigation at the present time.

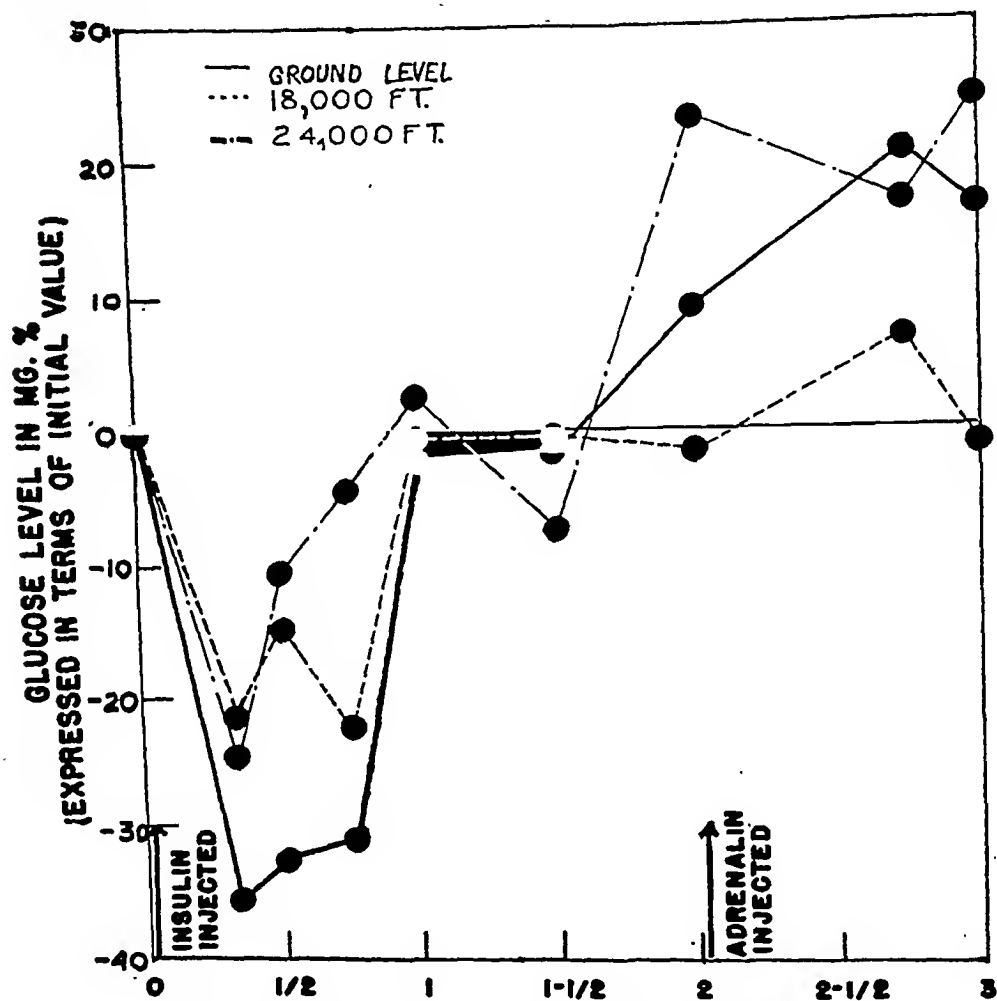


FIG. 2. MEAN INSULIN TOLERANCE [CURVES ON DOGS 1, 2 AND 3.

SUMMARY

Adult, male Dalmatian coach hounds were subjected to standard dextrose tolerance and insulin tolerance tests at ground level and at simulated altitudes of 18,000 feet and 24,000 feet. Differences in the responses to these tests were noted under the conditions of reduced barometric pressure.

The tolerance of the animals for intravenously administered dextrose was affected adversely by exposure to simulated altitude. This was manifested by a prolonged elevation of the blood sugar levels and by their failure to return

to normal fasting values in a three-hour period. This effect was much more pronounced at 24,000 feet than at 18,000 feet.

The insulin tolerance tests revealed a decreased insulin response in the animals exposed to simulated altitudes with no appreciable change in the time required for the return of the blood glucose level to the initial fasting level. The response to intramuscularly administered adrenalin was not as marked at the simulated altitudes as at ground level.

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HISTOCHEMICAL STUDY OF 'ALKALINE' PHOSPHATASE OF THE KIDNEY OF THE CASTRATED MOUSE AFTER STIMULATION WITH VARIOUS ANDROGENS¹

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Received for publication December 17, 1947

In previous reports it has been demonstrated that testosterone propionate (2) and many related steroids (3) produce a decrease in the 'alkaline' phosphatase of the kidney of the mouse. This report is an extension of these studies to several other androgens and a determination of the sites at which these changes occur as determined by the histochemical technic (4).

PROCEDURE

Male mice² of the Murray-Little dba strain were castrated under ether anesthesia at 16.0–19.5 grams body weight; one month later pellets of the various steroids³ were implanted subcutaneously (5, 6). The amount of steroid absorbed was increased by implanting two or more pellets and decreased by pellets composed of mixtures of the steroid hormone and cholesterol in the desired proportions (6, 7).

The food, Purina fox chow checkers (table 2) or Rockland checkers (table 1), was removed from the mouse cages 18 to 20 hours before autopsy and the mice were killed by decapitation. The kidneys were removed, a 2 mm. slice was cut transversely through the mid-section of one of the kidneys, weighed and placed in 15 ml. of cold redistilled acetone for histo-'alkaline' phosphatase determination (4, 8). The remainder was weighed, homogenized and the enzymes determined as previously described (3).

RESULTS

In vitro studies (table 1). Androstenedione during only 10 days of treatment was very effective in decreasing the 'alkaline' phosphatase of the kidney. Dehydroiso-androsterone and iso-androsterone, even though absorbed at a relatively high dose level, caused only a small increase in kidney size which was accompanied by a small increase in the enzyme. Androsterone produced a small and androstenedione a moderate decrease in the amount of enzyme per gram of tissue. Neither of these latter two compounds, however, was as effective as

¹ This investigation was aided by grants from the Josiah Macy Jr. Foundation. Parts of these data have been reported in the Josiah Macy Jr. Foundation Conference on Metabolic Aspects of Convalescence, fifth meeting, 136, and a review (1).

² The mice were provided by Dr. S. B. Warner of the Biological Station, Springville New York.

³ The steroids were provided by Ciba Pharmaceutical Products, Inc.

androstenedione which produced only a slightly greater increase in kidney size but a much greater decrease in enzyme activity.

17-methylandrostanol-17 α , one-3 produced an effect on the enzyme similar to that previously observed (3) when very potent renotrophic steroids, e.g., 17-methyl-testosterone, testosterone, etc. (cf. table 2) were used. At the lowest dose level, this compound produced an effect on the kidney weight and enzyme comparable to that obtained with androsterone.

The 'acid' phosphatase always increased (cf. 3) in amount at a rate equal to or slightly greater than the increase in kidney size (table 1).

TABLE 1. EFFECT OF STEROID HORMONES ON THE PHOSPHATASES OF THE KIDNEY OF THE CASTRATED MOUSE

TREATMENT	NO. MICE	NO. PELLETS	STEROID AB- SORBED	KIDNEY WGT.	PHOSPHATASES			
					Alkaline		Acid	
					Total per cent	Per gram per cent	Total per cent	Per gram per cent
10 days								
Δ^4 -Androstenedione-3,17	5	2	mgm. 6.8	mgm. 364	-39	-52	+21	-2
30 days								
Dehydroisoandrosterone	4	4	35.7	314	+16	+6	+36	+16
Isoandrosterone	4	4	28.2	330	+30	+12	+36	+15
Androsterone	4	4	13.0	348	+4	-13	+29	+4
Androstanedione-3,17	3	4	32.3	343	-15	-28	+14	-1
17-Methyl-androstanol-17 α ,one-3	3	1:4 ²	0.09	341	0	-15	+11	-7
	4	1:1 ²	0.45	476	-16	-49	+93	+14
	4	1	1.6	547	-40	-68	+97	+2
	4	2	3.3	566	-36	-68	+118	+9

¹ Change from the average values of 4 castrated control mice: Kidney weight = 284 grams; 'alkaline' phosphatase, total units = 116, units/gram = 401; 'acid' phosphatase, total units = 28, units/gram = 9.9 (cf. 3).

² Steroid: cholesterol composition of pellet.

*Histochemical studies.*⁴ The 'alkaline' phosphatase (black areas, fig. 1, 2, 3 and 4) was present in the proximal convoluted tubule, the cells lining Bowman's capsule and at the turn of Henle's loop. It was specially dense along the brush border (cf. 4). After castration, the cells in the proximal convoluted tubule became smaller (cf. 9) and the enzyme more concentrated (fig. 1 and 2). As the kidney increased in size under steroid stimulation these cells became larger and the enzyme began to disappear in a definite pattern. The decrease was

⁴ Charles Luttrell assisted in the determination and study of the experiments in table 1 and Dr. Victor Emmel assisted in the study of the experiments in table 2.

TABLE 2. TREATMENT AND WEIGHT OF MOUSE KIDNEY STUDIED FOR 'ALKALINE' PHOSPHATASE BY THE HISTOCHEMICAL TECHNIC¹

TREATMENT	NO. MICE	STEROID ABSORBED	KIDNEY
		mgm.	mgm.
Castrated.....	8		264
Normal.....	12		414
Testosterone.....	4	0.14	389
Testosterone.....	4	0.37	417
Testosterone.....	4	1.15	505
Testosterone.....	7	4.06	469
Testosterone.....	1	8.5	477
Testosterone.....	3	16.2	541
Testosterone propionate ²	4	0.20	392
Testosterone propionate ²	4	0.88	458
Testosterone propionate ²	5	2.33	490
Testosterone propionate ²	1	4.10	446
17-methyl testosterone ³	4	0.14	415
17-methyl testosterone ³	4	0.57	424
17-methyl testosterone ³	4	1.30	466
17-methyl testosterone ³	6	4.26	507
17-methyl testosterone ³	1	8.10	540
17-methyl testosterone ³	4	14.40	525
17-ethyl testosterone ⁴	2	1.75	257
Androstanol-17 α , one-3.....	5	0.65	424
Androstanol-17 α , one-3.....	2	6.4	518
Androstanol-17 α , one-3.....	4	7.1	505
Δ^4 -Androstenedione-3, 17.....	2	19.7	420
Androstanediol-3 α , 17 α	3	0.25	383
Androstanediol-3 α , 17 α	1	1.5	470
Androstanediol-3 α , 17 α	4	3.8	472
Androstanediol-3 α , 17 α	4	5.5	464
Androstanediol-3 α , 17 α	3	5.9	466
Androstanediol-3 α , 17 α , acetate-3.....	6	2.3	421
Androstanediol-3 α , 17 α , acetate-3.....	8	3.0	399
Androstanediol-3 β , 17 α	4	2.0	333

¹ The results obtained by *in vitro* homogenate studies have been reported (3).² Perandren.³ Metandren.⁴ Pregnynolone, lutocylol.

TABLE 2—Continued

TREATMENT	NO. MICE	STEROID ABSORBED	KIDNEY
		mgm.	mgm.
17-methyl androstanediol-3 α ,17 α	4	0.6	459
17-methyl androstanediol-3 α ,17 α	5	3.2	517
17-methyl androstanediol-3 α ,17 α	6	5.5	448
17-methyl androstanediol-3 α ,17 α	5	6.9	507
17-methyl androstanediol-3 β ,17 α	7	1.8	420
17-methyl Δ^5 -androstenediol-3 β ,17 α	4	2.4	347
Δ^5 -pregnenol-3 β ,one-20.....	1	4.6	305

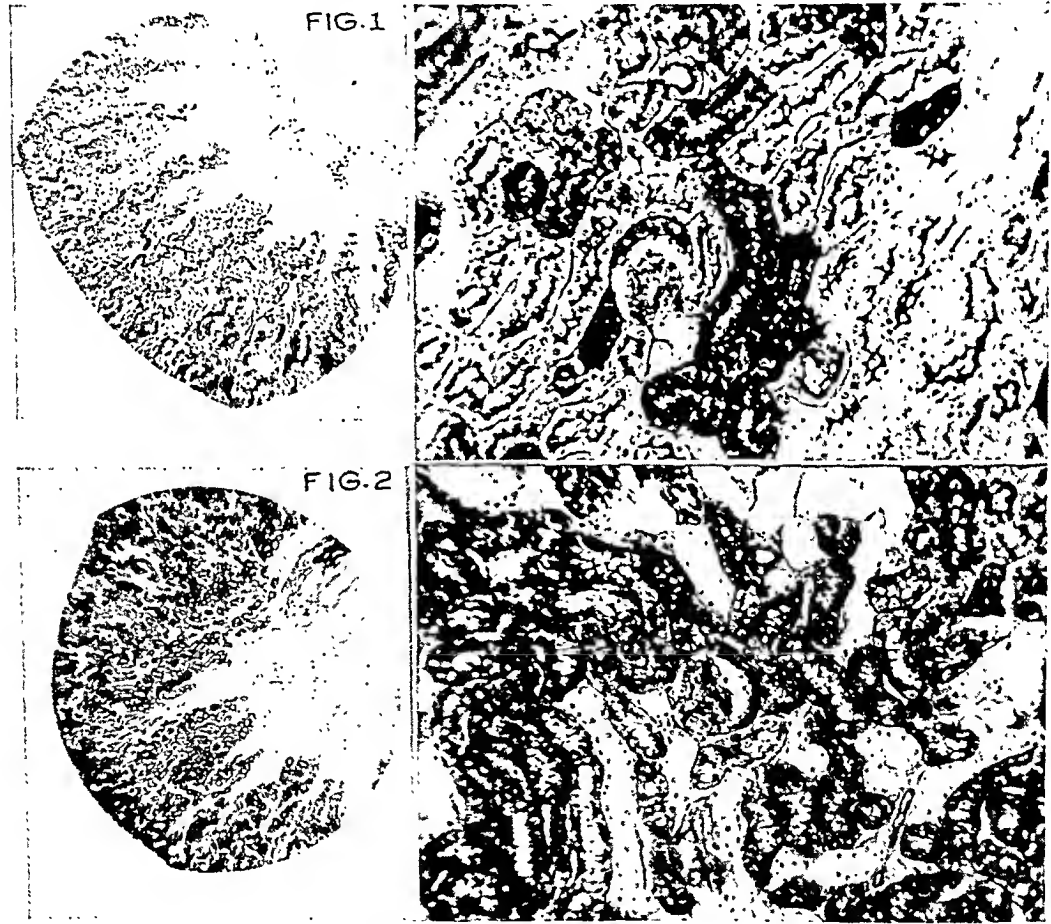


FIG. 1. KIDNEY OF A NORMAL MOUSE. Kidney weight = 419 mgm.; 'alkaline' phosphatase = 195 units/gram; A = 25 \times , B = 200 \times .

FIG. 2. KIDNEY OF A CASTRATED MOUSE. Kidney weight = 280 mgm.; 'alkaline' phosphatase = 414 units/gram; A = 25 \times , B = 200 \times .

progressive from the distal to glomerular end of the proximal convoluted tubule so that at maximal decrease in enzyme activity tubules of varying degrees of depletion were evident, e.g., testosterone (fig. 3) and 17-methylandrostanediol-3 α , 17 α (fig. 4). Furthermore, with depletion of enzymes from the distal end of the proximal convoluted tubule, the enzyme became slightly more concentrated

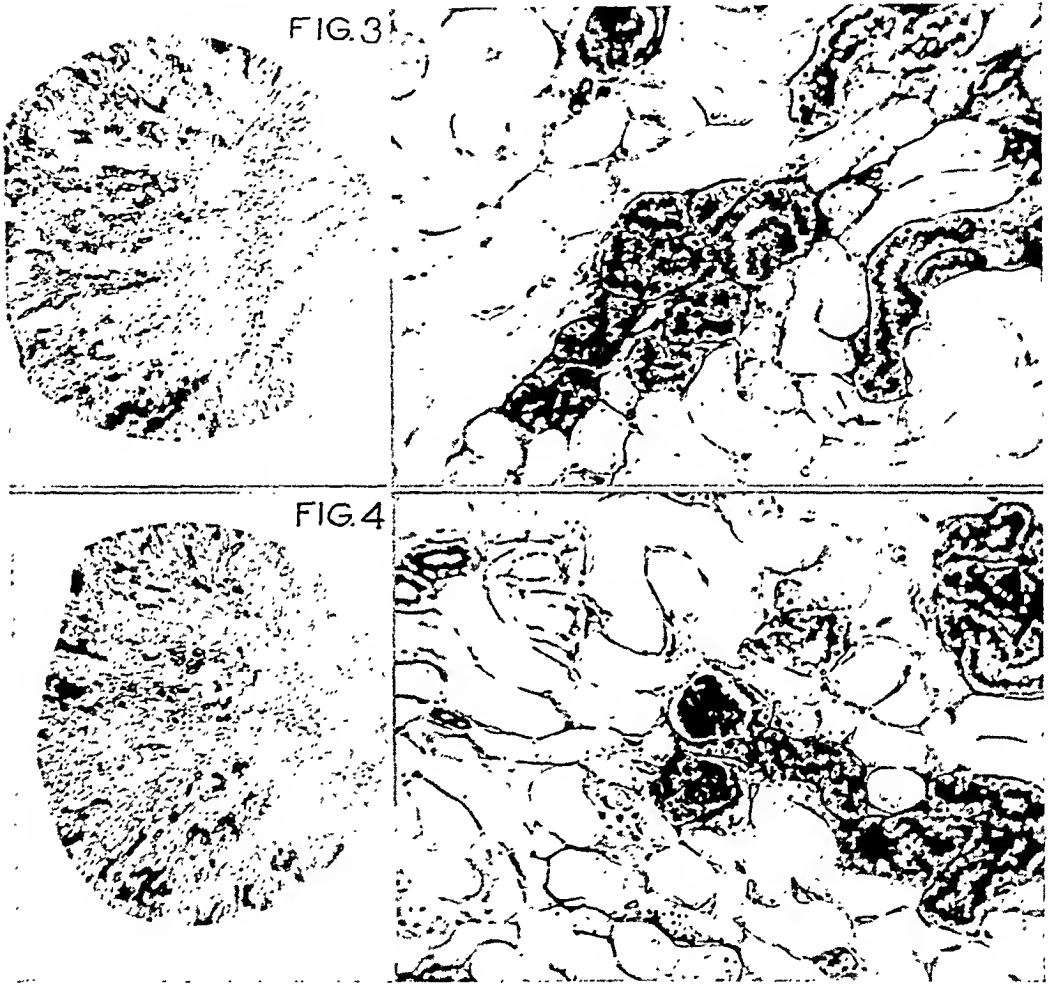


FIG. 3. KIDNEY OF A CASTRATED MOUSE implanted for 30 days with a pellet of 1:1 testosterone and cholesterol. Kidney weight = 477 mgm.; 'alkaline' phosphatase = 52 units/gram; A = 25 \times , B = 200 \times .

FIG. 4. KIDNEY OF A CASTRATED MOUSE implanted for 30 days with 4 pellets of 17-methylandrostanediol-3 α , 17 α . Kidney weight = 482 mgm.; 'alkaline' phosphatase = 63 units/gram; A = 25 \times , B = 200 \times .

at the glomerular end. The ability of the steroids to produce these effects paralleled their renotrophic activities.

The duodenum also was studied⁵ but no noteworthy changes were observed.

⁵ A report on the observation of 'alkaline' phosphatase in the Golgi apparatus has been made (10).

DISCUSSION

The results obtained with the use of the steroids of this study not only confirm the previous results (1, 2, 3) but also indicate that in the very early stages of the increase in kidney size, there is a slight increase in the 'alkaline' phosphatase. When, however, the androgens stimulate the kidney weight to greater than about 20 per cent, there is a progressive decrease in the enzyme which parallels the increase in kidney size. Furthermore, the initial increase in the 'alkaline' phosphatase is in agreement with the hitherto apparently anomalous small increases of this enzyme noted in the kidneys of rats (8, unpublished), guinea pig (unpublished) and hamster (unpublished) for in these animals the androgens produce less than a 20 per cent increase in kidney size.

The initial increase in the 'alkaline' phosphatase followed by a progressive decrease from the distal end of the proximal convoluted tubule toward the glomerulus with an increase in concentration near the glomerulus suggests that, if the enzyme is involved in the reabsorption of certain materials (11), then these substances are not only filtered through the glomerulus in smaller and smaller quantities but also that they are being reabsorbed as rapidly as possible under androgen stimulation for presumably anabolic processes (12).

SUMMARY

The distribution of the 'alkaline' phosphatase of the kidney of the mouse has been studied after treatment with 18 different steroids many of which were studied at several dose levels. There was a progressive decrease in the enzyme from the distal end of the proximal convoluted tubule toward the glomerulus with a slight increase in concentration at the glomerular end. These changes paralleled the increase in kidney size under steroid stimulation so that at maximum stimulation the kidney showed varying degrees of depletion of the enzyme in its nephrons.

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VITAMIN A, IODIDE AND THYROTROPIC HORMONE CONTENT OF THE ANTERIOR PITUITARY

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Received for publication December 9, 1947

Excessive intake of vitamin A reduces the secretion of thyrotropic hormone and also reduces the size of the thyroid and the metabolic rate (1-7). It is curious that excess vitamin A not only reduces the thyroid size but it also reduces the metabolic rate of hyperthyroid rats (7). If the reduction of the metabolic rate were brought about by thyroxine destruction, the lowered thyroxine level should increase the thyrotropic hormone secretion and thereby increase the size of the thyroid; but the size of the thyroid has not increased but decreased. A possible explanation of this unexpected effect is that vitamin A, by virtue of its double bonds, combines with the iodine of the thyroxine and this vitamin-iodine compound then behaves, in vitamin-antivitamin fashion, as thyroxine would in depressing thyrotropic hormone secretion. As the combination was expected to be between vitamin A and iodine of the thyroxine, a similar effect might be expected to be produced by feeding iodide together with vitamin A, since iodide given orally is said to be oxidized in the body to iodine unless such reducing agents as goitrogens are administered simultaneously (7a).

In iodine deficiency the thyroxine synthesis is decreased and this resultant low thyroxine level stimulates the secretion of thyrotropic hormone, which enlarges the thyroid gland. Iodide fed to animals increases the protein-bound iodine in the pituitary, which acts as thyroid hormone in inhibiting the formation of the thyrotropic hormone. This decrease in thyrotropic hormone leads to decreased activity of thyroid and lowers the basal metabolic rate (8). This paper reports a study of the effect of feeding excess vitamin A together with iodide on the thyrotropic hormone content in rats.

EXPERIMENTAL

Experiments were conducted with four groups of male albino rats; nine rats were placed in each group. One group served as control; the second group was fed 30,000 I. U. vitamin A daily in the form of percomorph oil; the third group was fed one gram potassium iodide daily; the fourth group was fed the above amounts of both vitamin A and potassium iodide. After 18 days on these diets, the rats were sacrificed and their thyroids, adrenals and pituitaries weighed in a precision balance. The results are shown in table 1.

The pituitaries were assayed for thyrotropic hormone on day-old chicks. Water suspensions of pituitaries were prepared by rubbing the gland in an agate

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mortar and made up to volume with distilled water, so that there were 10 mgm. of fresh pituitary tissue in one ml. of suspension. Five groups of day-old chicks were used; one group served as control while the other four groups were injected with the pituitary suspension of the above four groups of experimental rats for a period of four days. The chicks were sacrificed on the fifth day of the experiment and their thyroids dissected out and weighed. The results are shown in table 2.

The chick thyroids, prepared histologically, were stained with hematoxylin and

TABLE 1. EFFECT OF POTASSIUM IODIDE AND VITAMIN A ON THE ENDOCRINE ORGANS OF THE RAT

GROUP	NO. OF RATS	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	GAIN IN WEIGHT PER DAY	THYROID WT. PER 100 GRAMS BODY WT.	ADRENAL WT. PER 100 GRAMS BODY WT.	PITUITARY WT. PER 100 GRAMS BODY WT.
		grams	grams	grams	mgm.	mgm.	mgm.
Control.....	9	89	167	4.3	6.56	11.89	3.46
Fed one gram KI daily..	9	89	150	3.4	5.89	11.74	3.69
Fed 30,000 I.U. vitamin A daily.....	9	93	139	2.6	4.74	16.70	4.22
Fed vitamin A (30,000 I.U.) and KI (1 gram) daily.....	9 ¹	86	112	1.4	6.61	18.44	4.78

¹ Three of the rats died during the experiment.

TABLE 2. ASSAY OF THYROTROPIC HORMONE BY CHICK AND HISTOLOGICAL METHODS (4 mgm. pituitary tissue injected)

GROUP	NO. OF CHICKS	BODY WEIGHT	THYROID WT. PER 100 GRAMS BODY WT.	HEIGHT OF EPITHELIA
		grams	mgm.	micra
Control.....	11	47	5.99	3.78
Control rat pituitary group.....	11	47	11.23	5.88
KI-fed rat pituitary group.....	11	48	10.91	—
Vitamin A-fed rat pituitary group...	11	51	9.82	5.04
Vitamin A- and KI- fed rat pituitary.....	7	47	11.83	5.78

eosion. The diameters of the epithelia of the thyroid acini were measured and the results are presented in table 2.

DISCUSSION

Table 1 indicates that on the high vitamin A diet the daily gain in body weight is 2.6 grams as contrasted to 4.3 grams in the control group and that the thyroid weight on the high vitamin A diet is significantly lower and the adrenal weight significantly higher than on the control diet. The pituitary weight also increased, but the iodide-fed group shows a smaller thyroid weight than the control group. This is probably due to the fact that iodide after *in vivo* oxidation to

iodine causes iodination of anterior pituitary tissue protein (8) or of proteins elsewhere, leading to the formation of thyroxine, which depresses the formation of thyrotropic hormone by the anterior pituitary, and consequent decrease in thyroid size. There is no change in the size of adrenal or pituitary. When both vitamin A and potassium iodide are fed, the combination becomes rather toxic; three of the nine rats died during the experimental period. Toxicity was not due, however, to iodism, as the same amount of iodide fed to other rats did not produce toxic symptoms. The toxic condition is also shown by the increase in adrenal size, with no concomitant change in thyroid size.

Both iodide and vitamin A have been found to lower the thyrotropic hormone secretion (table 2). The metabolic rate-lowering effect of vitamin A in normal rats can be explained by the thyrotropic-depressing effect of the assumed formation of iodized vitamin A, as previously explained, but not in rats made hyperthyroid by injecting thyroxine. Therefore, vitamin A must act in the peripheral end organs by preventing the action of thyroxine on the enzyme systems on which thyroxine acts. As vitamin A lowers the thyrotropic hormone content of the pituitary (table 2), it was assumed that the effect must be produced by the vitamin A-thyroxine compound which acts as thyroxine on the anterior pituitary. There is no direct evidence that such a compound is formed in the body. *In vitro*, however, there is spectroscopic evidence for such a combination with carotene and thyroxine (9, 10). As vitamin A retains most of the chemical properties and also the lyophobic characteristics of its parent chemical substance, carotene, it is expected that, like carotene, it will be iodinated at some of its double bonds. This may be made possible by feeding iodide when the iodide is oxidized into elementary iodine in the body. The toxicity of vitamin A in combination with iodide feeding also shows that vitamin A combines with iodine liberated *in vivo* from iodides. Thyroxine-iodine may similarly combine with vitamin A. But with potassium iodide feeding and, therefore, with *in vivo* liberation of elementary iodine, since vitamin A does not lower thyrotropic hormone secretion by the anterior pituitary, it must be assumed that the effect on the pituitary is exercised by vitamin A alone and not by the vitamin A-thyroxine compound if such a compound is formed. It would appear that part of the vitamin A supplied neutralizes some of the metabolism-stimulating effect of thyroxine while the remaining of the vitamin supplied, which is not combined with thyroxine, acts on the pituitary and lowers the thyrotropic hormone secretion.

The results reported in this paper appear to be the specific effect of vitamin A and cannot be due simply to nonspecific toxicity as under toxic conditions; though the adrenal gland is enlarged, there is no evidence for a decrease in thyroid size. Again vitamin A depresses the hypermetabolism produced by injection of thyroxine, but not of dinitrophenols. Neither can the results be due to failure to gain body weight, for the difference between the groups is more than can be explained by a simple change in body weight. It will be noted in table 1 that as the body weight gain decreases progressively in the four groups of rats, thyroid weight does not correspondingly decrease, but in the last group, with the least body weight gain, the thyroid weight is comparable to that of the control rats.

The way in which vitamin A lowers thyrotropic hormone secretion (table 2) is not understood. It has been assumed that vitamin A may compete with thyroxine on the metabolic systems and thus lower the metabolic effect. But as there is no similarity between the chemical configurations of vitamin A and thyroxine, this assumption cannot be proved.

From these experiments it is concluded that vitamin A reduces the thyroid size by depressing the secretion of thyrotropic hormone by acting directly on the anterior pituitary and not by forming an iodide compound.

SUMMARY

1. Vitamin A in large doses (30,000 I. U. per day per rat) decreases the thyroid size. This effect is apparently produced by lowering the secretion of thyrotropic hormone of the anterior pituitary. Excess vitamin A also increases the size of the adrenal gland.

2. Potassium iodide slightly lowers the thyroid size and depresses the thyrotropic secretion, or inactivates the thyrotropic hormone.

3. Iodized vitamin A has no influence on thyroid size or on the thyrotropic secretion, confirming the conclusion that vitamin A lowers the thyrotropic secretion directly and not by compound formation with thyroxine.

4. Thyrotropic hormone has been assayed by the response in day-old chicks and confirmed histologically by measuring the height of epithelia in thyroid acini.

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SHRINKAGE OF LYMPHATIC TISSUE IN RATS FOLLOWING INJECTIONS OF INSULIN

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Received for publication January 1, 1948

It is well established that acute injury of the body initiates a chain of reactions by which the anterior pituitary stimulates the adrenal cortex to secrete a hormone which in turn causes dissolution of lymphocytes in lymphatic tissue, releasing protective substances, this constituting a defense reaction or adaptation syndrome. Consistent with the interesting hypotheses of Vogt (1) and of Long and Fry (2), it may be postulated that following acute bodily injuries, the prompt response of the sympathetic nervous system in releasing epinephrine from the medulla is followed by the slower but more sustained activity of the cortex—a coordinated process which gives meaning to the anatomic juxtaposition of cortex and medulla. In support of this concept, there may be gathered from the literature various data concerning separate phases of this series of reactions. It has been shown that following injections of epinephrine in animals, the cortex hypertrophies (3); adrenal cortical secretion occurs (1); and the cortex loses its lipoids (2), which is now known to be indicative of discharge of hormone. Selye (4, 5) has included epinephrine injections as one of the many damaging agents causing the 'alarm reaction' with resultant reduction in lymphatic tissue. However, adrenal demedullation does not prevent the fall in adrenal cholesterol after exposure to cold (6) and Long (7) states "the hypothesis that sympathetic stimulation (and release of epinephrine) is an essential link in the pituitary-adrenal cortex activation is at present not completely established." It seems of interest, therefore, to learn more about the factors which start this response.

Experiments were planned to see if repeated large doses of insulin, known to result in stimulation of the adrenal medulla through the sympathetic nervous system, would release enough endogenous epinephrine to reproduce the effect of injections of exogenous epinephrine on lymphatic tissue. It is known that repeated doses of insulin may cause enlargement of the adrenal cortex. The literature on this subject has been reviewed (8), but data were not found on whether or not insulin injections caused shrinkage of lymphatic tissue. Latta and Henderson (9) in 1937 found that repeated insulin injections into rats caused transient reduction in numbers of lymphocytes in the blood, and hyperplasia of reticular cells and lymphocytes in lymph nodes with degenerated cells in lymph nodes, which in the light of our present knowledge we would attribute to adrenal cortical activity. Weights of lymphatic tissues were not given and it was not suggested at the time that the destruction of lymphocytes might be, not a direct action of insulin, but a result of a response provoked by insulin in other organs.

EXPERIMENTS

A group of nine rats were injected repeatedly with epinephrine to establish data as a standard of comparison for the effect of insulin. They were injected two or three times daily with 1:1000 solution diluted so as to give individual doses varying from 0.010 mgm. to 0.070 mgm. per 100 grams body weight, over a period of three to seven days. Controls were nine rats either litter-mates of the same sex or rats of equal body weight, either uninjected or injected with water to control the effect of trauma.

Another group of 16 rats were given insulin injections, usually in ascending doses, twice daily, varying from 0.2 to 2.5 units per 100 grams body weight over periods of three to seven days, in order to cause repeated severe hypoglycemia. They were not fasted except in some cases for two hours after the injection, but were allowed food so as to avoid the effect of inanition on the adrenal cortex and lymphatic tissue. Controls were 16 rats, either litter-mates or rats of the same body weight and same sex and they were either not injected or injected with water. With ascending doses some of the rats were able to survive very large doses, presumably as a result of adrenal cortical hypertrophy. This may be the explanation of the increased tolerance of the rabbit to huge doses of insulin described in previous experiments (10). When necessary, glucose was given by mouth to relieve hypoglycemia. Occasionally it was necessary to inject glucose intraperitoneally to save the life of the rat, and then the control rat was similarly injected. At appropriate intervals the rats were either killed with anesthetic or allowed to die of hypoglycemia. Tissues were weighed rapidly to the nearest mgm. Some injected rats lost weight, so the final body weights varied slightly from the controls.

As rats of different ages and sizes were used, there is great variation in the weights of the different organs (table 1). Each injected rat was paired with a rat of similar body weight, and the purpose was not to compare absolute weights but to determine in the case of each pair whether or not the injected rat showed alteration from the control. These alterations are expressed as percentage increase or decrease in table 1 and figure 1. It may be seen that with few exceptions the lymphatic tissue shrinks as the result of the injections and the adrenals enlarge. Since much evidence in the literature indicates that adrenal hypertrophy and secretion is always accomplished by way of the pituitary, the insulin and epinephrine injected were presumably effective in stimulating the pituitary. In the instances in which adrenal weights were decreased, probably the dosage had been so intense as to lead toward adrenal exhaustion with consequent lessened shrinkage of lymphatic tissue.

An attempt was made to see if adrenalectomy would abolish the effect of insulin on lymphatic tissue. Adrenalectomized rats were kept in good condition by NaCl in the drinking water. Two weeks or longer after operation they were injected with repeated doses of insulin. Difficulties were encountered because of the well known fact that adrenalectomized animals are extremely sensitive to insulin. Many died in hypoglycemia soon after receiving minute doses of

TABLE 1. COMPARISON OF INJECTED RATS WITH CONTROLS

	NO. OF RATS	FINAL BODY WEIGHT, MEAN	THYMUS, MEAN	MESENTERIC LYMPH NODES, MEAN	ADRENALS, MEAN	PERCENTAGE CHANGE IN WEIGHT OF INJECTED RATS AS COMPARED WITH CONTROLS		
						Thymus, mean	Mes- enteric lymph nodes, mean	Adrenals, mean
Controls.....	9	<i>gms.</i> 248	<i>gms.</i> 0.35 ±0.059 ¹	<i>gms.</i> 0.365 ±0.044 ¹	<i>gms.</i> 0.039 ±0.003 ¹	<i>per cent</i> -39%	<i>per cent</i> -13%	<i>per cent</i> +9%
Injected with epine- phrine.....	9	241	0.234 ±0.054	0.306 ±0.031	0.043 ±0.003			
Controls.....	16	203	0.354 ±0.028	0.314 ±0.007	0.038 ±0.003	-13%	-19%	+8%
Injected with in- sulin.....	16	192	0.306 ±0.026	0.244 ±0.013	0.054 ±0.003			

¹ Standard error.

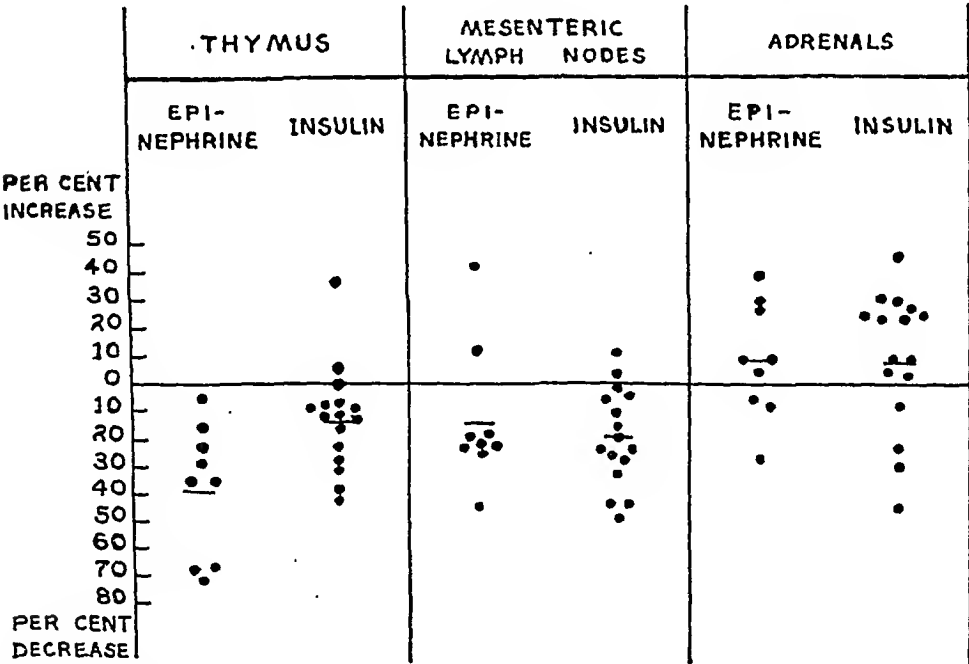


FIG. 1. PERCENTAGE CHANGE IN WEIGHTS of organs of rats injected with epinephrine and with insulin in comparison with controls.

insulin such as 0.025 units per 100 grams body weight. It seemed impossible to maintain hyoglycemia by any dose of insulin for periods comparable to those in the series of intact rats. The weights of lymphatic tissue were so variable that no conclusions could be drawn. Attempts at denervating the adrenal

medulla and leaving the cortex intact resulted in so much scarring in the region of the adrenal that the results were not thought to be valid. It was therefore not possible to prove the rôle of the adrenal medulla or cortex in these experiments.

SUMMARY

The experiments give evidence that, in rats, exposure to repeated injections of insulin causes reduction in weight of lymphatic tissue. Since insulin hypoglycemia is known to result in discharge of endogenous adrenalin, it is possible that secretion of the adrenal medulla has activated the adrenal cortical-lymphatic tissue reaction.

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INTERRELATIONSHIPS OF SPERMATOOZOA COUNT, HYALURONIDASE TITER AND FERTILIZATION

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Received for publication December 29, 1947

The problem of fertilization in animals and man has engaged the attention of a number of workers and the knowledge accumulated has brought into focus three factors which appear to be of significance in the process of fertilization. These are the preparation of the ovum prior to sperm penetration, the influence of spermatozoa numbers and the relation of the enzyme hyaluronidase to fertilization.

The cumulus cells of mammalian ova have been described as being cemented together by a viscous substance (1). It has been suggested that this cementing substance is hyaluronic acid and that it is rendered non-viscous by the action of hyaluronidase present in normal semen, thus allowing dispersal of the cumulus cells in the process of fertilization (2, 3). The amount of hyaluronidase present in semen has been reported as varying positively in a linear fashion with the number of spermatozoa (4, 5).

Many attempts have been made to determine the minimal and optimal sperm concentrations required for fertilization in various species (6, 7, 8) but, in general, there has been little agreement in comparable situations.

It is the purpose of this paper to report our studies on the quantitative interrelationships of the spermatozoa count, hyaluronidase titer and percentage fertilization in cattle and to indicate their probable importance to the problem of fertilization as a whole.

METHODS

Seminal specimens were collected from individual bulls and part of each specimen was set aside for spermatozoa count and hyaluronidase assay, which were made within twenty hours after collection. Specimens were diluted 1:55 with a sterile diluent consisting of equal parts of egg yolk and distilled water containing 36 grams of sodium citrate and 3 grams of sulfanilamide per liter. Diluted seminal specimens were kept cold during transit to 45 inseminators throughout Ohio. The majority of inseminations were accomplished within 48 hours of semen collection and the remainder within 72 hours. One cc. of diluted semen was used in each insemination.

The criterion for successful fertilization was the failure of the cattle owner to request another insemination (nqn-return). Since three services are guaranteed

¹ This work was supported in part by a grant from the Ohio Purebred Dairy Cattle Association.

The authors wish to express their appreciation to Dr. W. E. Krauss and Dr. Scott T. Sutton of the Department of Dairy Husbandry and to R. H. Kellogg and J. Curran of the Central Ohio Breeding Association for their assistance.

by the original fee, unsuccessful inseminations have invariably been followed by further requests (return). For any one cow, only the fertilization results of a maximum of two inseminations were recorded. Beyond this number the fertility of the cow and the assurance of another request for semen were open to question. A period of three months from the time of insemination was allowed to elapse before a non-return was considered valid.

Spermatozoa counts. To minimize the 'normal' counting chamber and pipette error, all spermatozoa counts were made on one Spencer 'bright-line' haemocytometer and with the same two red cell dilution pipettes. A diluting fluid containing 5 per cent sodium bicarbonate and 1 per cent phenol was used. In making a count the semen sample was thoroughly mixed and drawn to the 0.5 mark, resulting finally in a dilution of 1:200. The actual determination was made by filling the counting chamber and counting the four corner and center squares of the customary red cell area. Seven zeros were added to the total number of sperm in the five squares to give the count per cc.

Individual counts were repeated 15 to 20 times in an effort to determine the amount of variance between counts. It was found that all counts for a single specimen fell within ± 18 per cent of the mean count. Werthessen *et al.* (4) arrived at a ± 20 per cent range for sperm count variance. Magath *et al.* (9) found the error of a single estimate of the erythrocyte count to be ± 16 per cent. Apparently, the greater variance with spermatozoa is due to some property of the semen, possibly its greater viscosity. All sperm counts made in the experimental work were an average of four determinations. Sperm counts made were total cell counts; motility and morphology studies could not be accomplished under the conditions of semen collection.

Hyaluronidase assay. Hyaluronidase assays were made turbidimetrically (5). This method is based on the observation that pure hyaluronate at pH 4.2 gives a stable colloidal suspension when mixed with dilute blood serum, whereas, hyaluronate depolymerized by hyaluronidase action remains clear. The hyaluronidase unit is designated as the turbidity-reducing unit (TRU) and is defined as the amount of hyaluronidase which will reduce the turbidity produced by 0.2 mgm. of hyaluronic acid under given conditions to the equivalent turbidity produced by 0.1 mgm. of hyaluronic acid in 30 minutes at 37°C. Turbidity measurements were made with an Evelyn photoelectric colorimeter using a 660 mμ color filter. Colorimeter readings plotted against varying hyaluronidase concentrations produced an S-shaped curve but in the region between 0.2 mgm. and 0.1 mgm. the curve was almost a straight line. We have found that greater rectification of the linear function was obtained when turbidity readings were plotted against the logarithms of the hyaluronidase dilutions rather than the dilutions themselves. Therefore, within the test range of hyaluronic acid and in utilizing the above modification, decrease in turbidity is a linear function of enzyme concentration. All hyaluronidase values were calculated in this fashion.

It was found that when different batches of horse serum or the same batch at successive intervals of approximately a week, whether previously diluted or not, was used in the turbidimetric test procedure, a single semen specimen gave

significantly different hyaluronidase titers. Upon further investigation it became apparent that the varying titers were associated with inequalities in the turbidity ranges between the 0.2 mgm. and the 0.1 mgm. standards caused by the serum-hyaluronate complex. Large turbidity ranges caused lower values and, conversely, small ranges gave increased values. This relationship, however, was not strictly linear and it did not prove feasible to obtain a correction factor for varying sera. To obtain comparable hyaluronidase values, therefore, only sera exhibiting turbidity ranges of 6.0 ± 1 Evelyn galvanometer scale divisions were used in obtaining the enzyme titers.

The enzyme substrate, pure sodium hyaluronate, was prepared from human umbilical cords according to the method of McClean (10) as modified by Rogers (11).

EXPERIMENTAL RESULTS

A total of 2992 inseminations were made in 2092 cows. The comparative success of first and second inseminations are given in table 1.

TABLE 1. FERTILIZATION RESULTS WITH FIRST AND SECOND INSEMINATIONS

	TOTAL	1ST INSEMINATION	2ND INSEMINATION
Fertilizations.....	1618	1192	426
Non-fertilizations.....	1374	900	474
Per cent fertilizations.....		57.0	47.3
Per cent non-fertilizations.....		43.0	52.7
Inseminations.....	2992		
Cows.....	2092		

It will be seen from table 1 that 57 per cent of the cows under observation were successfully fertilized by the first insemination while 47.3 per cent of the remainder were fertilized by the second insemination. Both fertilization percentages lie within the usual range of successful fertilizations obtained by most insemination groups. In view of the fact that variations in the fertilizing capacities of different semen specimens and in appropriate insemination time and technique are normally operative, it can be concluded that only a relatively few cows can be suspected of sterility and that the animals used represented as a whole a statistically appropriate sampling for the purpose at hand.

The data in table 2 are arranged in ascending order of percentage fertilizations (C) which are based on the proportion of non-returns for the particular semen specimen. Spermatozoa counts (A) are given in millions of sperm per cc. while hyaluronidase activity (B) is measured in turbidity-reducing units per cc. There are a total of 49 individual semen specimens from 25 bulls for which data are listed.

The results of statistical analysis of the above data are given in table 3. It will be seen that the correlation coefficient between sperm count and hyaluronidase titer is $+0.27891$, the t value for this coefficient being 1.991. Since the level

TABLE 2. SPERM COUNT, HYALURONIDASE TITER AND PERCENTAGE FERTILIZATION
FOR INDIVIDUAL SEMEN SPECIMENS

SPERM COUNT IN ML./CC. (A)	HYALURONIDASE TRU/CC. (B)	NON-RETURN	RETURN	TOTAL RETURN, NON-RETURN	PERCENTAGE FERTILIZATION (C)
560	2933	20	60	80	25.00
1350	3673	12	25	37	32.43
1310	2956	20	40	60	33.33
400	1978	7	12	19	36.84
1090	3378	6	10	16	37.50
600	4333	23	38	61	37.70
470	1173	19	29	48	39.58
1120	2160	37	55	92	40.22
940	2167	19	25	44	43.18
470	1400	36	46	82	43.90
540	2200	32	40	72	44.44
1520	1643	27	32	59	45.76
1560	1956	33	39	72	45.83
410	1341	37	39	76	48.68
1130	3200	28	29	57	49.12
540	1278	29	28	57	50.88
1780	4200	22	21	43	51.17
1220	2986	13	12	25	52.00
1660	2134	26	24	50	52.00
1080	1190	44	39	83	53.01
690	1613	12	10	22	54.55
1590	1422	52	43	95	54.74
1520	1991	11	9	20	55.00
570	1750	25	20	45	55.56
2440	2533	29	23	52	55.77
700	2867	50	37	87	57.47
1060	2107	34	25	59	57.63
860	2444	36	25	61	59.02
1200	1369	37	25	62	59.68
760	1567	6	4	10	60.00
490	1583	29	19	48	60.42
600	1213	28	18	46	60.87
770	1956	42	27	69	60.87
1020	1422	38	24	62	61.29
1150	1511	39	24	63	61.90
780	1417	43	26	69	62.32
710	831	43	26	69	62.32
1240	3289	50	30	80	62.50
1480	2883	43	25	68	63.24
1090	1700	40	23	63	63.49
1730	3209	41	23	64	64.06
2730	2850	38	21	59	64.41
1890	1333	50	27	77	64.94
1380	1200	65	34	99	65.66
1020	1894	49	25	74	66.22
1320	1389	47	22	69	68.12
1100	1400	15	6	21	71.43
1200	3100	33	13	46	71.74
940	2167	26	10	36	72.22
53780	104289	1618	1374	2992	2660.01

N = 49.

of significance $P = .05$ has a t value less than 1.991, it was concluded that r_{AB} is statistically significant. The same conclusion is reached for $r_{BC} = -.32290$ whose t value lies between the $P = .02$ and $P = .01$ levels of significance. However, the correlation coefficient of sperm count with fertility $r_{AC} = +.24436$ has a t value between the $P = .1$ and $P = .05$ levels and hence cannot be considered significant.

All equations derived in this paper have been adjusted for values obtained from undiluted semen specimens. For computational use with diluted semen, appropriate changes in constants must be made for each particular dilution. Three regression equations (I, II, III) are listed in table 3 from which corresponding values of hyaluronidase titer and percentage fertilization can be obtained by inserting in the equations the respective values of sperm count or hyaluronidase titer. Percentage fertilization values derived from the hyaluronidase substitution equation will have a greater reliability than those derived from the sperm

TABLE 3. ZERO ORDER REGRESSION EQUATIONS AND COEFFICIENTS OF CORRELATION

ZERO ORDER REGRESSION EQUATIONS		STANDARD ERROR OF ESTIMATE
I	$B = 1617.36 + 465.57 \times 10^{-3}(A)$	$S_B = 806.14$
II	$C = 48.37 + 539.51 \times 10^{-5}(A)$	$S_C = 10.72$
III	$C = 63.34 - 425.45 \times 10^{-5}(B)$	$S_C = 10.46$
CORRELATION COEFFICIENT		t VALUE
$r_{AB} = +.27891$		1.991
$r_{AC} = +.24436$		1.728
$r_{BC} = -.32290$		2.339
LEVEL OF SIGNIFICANCE		CORRESPONDING t VALUE
$P = .05$		1.960
$P = .02$		2.326
$P = .01$		2.576
$P = .001$		3.291

count substitution equation due to the smaller standard error of estimate of the former and the fact that r_{AC} is not significant.

In deriving our statistical results thus far we have assumed an oversimplified condition. For instance, in obtaining the coefficient of correlation between sperm count and fertility we have assumed that no other influencing factors were present. Actually, we know that fertilization is also affected by hyaluronidase titer and in a negative fashion. Similarly, in determining the relationship between hyaluronidase and fertilization, the influence of sperm count must be taken into consideration. Thus, it is important to know the correlation between fertilization and sperm count when the influence of hyaluronidase titer is held constant and, similarly, the correlation between fertilization and hyaluronidase when sperm count is held constant. Fortunately, in being able to obtain the coefficient of net (partial) correlation, we possess a means of obtaining these ends. The coefficient of net correlation for sperm count and percentage fertilization when

the influence of hyaluronidase titer is held constant ($r_{AC.B}$) turns out to be $+0.36730$ with $t = 2.678$ (table 4). Correspondingly, $r_{BC.A} = -0.41999$ with $t = 3.139$. Both of these coefficients are highly significant as their t values indicate.

In obtaining regression equations II and III for percentage fertilization we were guilty of incompleteness as well as over-simplification. It is desirable to obtain a regression equation for percentage fertilization which would take into account the fact that fertilization is a function of hyaluronidase titer and of sperm count, both variables acting upon it simultaneously and in opposite directions. This equation is obtained by deriving a multiple regression equation for percent-

TABLE 4. MULTIPLE REGRESSION AND NET CORRELATION RESULTS

COEFFICIENT OF NET CORRELATION		t VALUE
$r_{AC.B} = +.36730$		2.678
$r_{BC.A} = -.41999$		3.139
MULTIPLE REGRESSION EQUATIONS		
IV	$C_1 = 799.08 \times 10^{-5}(A) - 557.54 \times 10^{-5}(B)$	
V	$C = 57.38 + 799.08 \times 10^{-5}(A) - 557.54 \times 10^{-5}(B)$	
STANDARD ERROR OF ESTIMATE		
$S_{C.AB} = 9.73$		
COEFFICIENT OF MULTIPLE CORRELATION		t VALUE
$R_{C.AB} = +.43890$		3.313

age fertilization, with sperm count and hyaluronidase titer as the two independent variables:

$$C_1 = 799.08 \times 10^{-5}(A) - 557.54 \times 10^{-5}(B) \quad \text{IV}$$

$$C = 57.382 + 799.08 \times 10^{-5}(A) - 557.54 \times 10^{-5}(B) \quad \text{V}$$

where A is millions of sperm/cc. and B is hyaluronidase titer in TRU/cc.

Equation IV contains the net coefficients of estimation, indicating the relative simultaneous effect of sperm count and hyaluronidase titer on fertilization. Equation V is the actual predictive equation for percentage fertilization. The standard error of estimate for V ($S_{C.AB}$) is 9.73. A comparison of this figure with the values of $S_C = 10.720$ and $S_C = 10.463$ which were obtained with equations II and III denotes the increased reliability of values obtained with equation V.

Finally, the derivation of one more coefficient is highly instructive. The coefficient of multiple correlation $R_{C.AB}$ is an indicator of the proportion of the total variation in percentage fertilization which is being measured by the multiple regression equation. $R_{C.AB}$ turns out to be $+0.43890$ with a t value of 3.313, indicating that this coefficient is highly significant statistically. The difference

between the square of the multiple correlation coefficient ($R^2_{C,AB} = .22385$) and unity indicates the proportion of the total variation which is accounted for by factors other than sperm count and hyaluronidase titer. That these factors represent the major portion of the total variation is readily understandable in view of the fact that successful fertilization also depends upon insemination occurring during the short estrus period, the use of uniformly appropriate insemination techniques in the field and the physiological fitness of the individual cow for pregnancy during the first three months following conception.

DISCUSSION

With the demonstration that hyaluronidase disperses the cumulus cells of mammalian ova and the consequent implication of the enzyme in the process of fertilization, the hypothesis has been advanced that millions of spermatozoa are required for the fertilization of a few ova not to assure the chance meeting of a sperm and ovum but rather to supply a sufficient concentration of hyaluronidase as a prerequisite for fertilization in the immediate environment of the sperm and ovum (7). This theory is further strengthened by the recent finding of more direct evidence indicating that the spermatozoa themselves produce the hyaluronidase content of semen and that the amount of hyaluronidase present is positively correlated with the number of spermatozoa (12, 13). A corollary to the theory, likewise, has been inferred by most investigators of the problem that seminal specimens containing greater amounts of hyaluronidase possess greater fertilizing capacities and that addition of the enzyme to semen in cases of oligospermia will increase the chances of fertilization.

In accord with the above observations, we have found hyaluronidase titers in cattle to be positively correlated with spermatozoa counts. Our results indicate, however, that above some hyaluronidase threshold value for fertilization, which at present remains obscure, increased amounts of hyaluronidase are associated with decreased fertilizing capacities. Thus hyaluronidase titer and percentage fertility have a net correlation coefficient of $-.41999$, a highly significant figure. A physiological explanation of this negative effect cannot be demonstrated with the available knowledge but an excess of the enzyme might produce an effect undesirable for fertilization on the ovum itself. In this respect it is interesting to note that Pincus and Enzmann (1) observed that rabbit semen which dispersed the cumulus cell mass of ova most speedily and had the greatest number of sperm per cmm. completely dissolved the ova after 24 hours.

It has been observed by several investigators that beyond a particular minimal range, increase in the number of spermatozoa used for insemination yields progressively higher percentages of fertilizations, until an optimal count is reached. Apparently beyond this optimal spermatozoa count, no further increase in the proportion of fertilizations accomplished is noticeable. The latter situation is the one encountered with normal bull semen as is demonstrated by the fact that the correlation of percentage fertilization with sperm count ($r_{AC} = +.24436$) which we obtained was not significant. However, since hyaluronidase titer is positively correlated with sperm count and, beyond a particular minimal value, progressive

amounts of hyaluronidase are associated with decreased fertilization percentages, it may well be that numbers of spermatozoa, greater than the so-called optimal count, may still be capable of producing higher fertilization percentages but that their influence is nullified by the associated increase in hyaluronidase content. Indeed just such a condition would seem to be operative. When the influence of hyaluronidase content is kept constant, the correlation between percentage fertilization and sperm count becomes highly significant ($r_{ACB} = +.36730$). Evidently the concept that large numbers of sperm serve to increase the mathematical chances of a sperm meeting the ovum must be at least in part retained unless another as yet unidentified factor, associated quantitatively with spermatozoa in fertilization, is assumed.

Clearly, any examination of the fertilizing capacity of semen must take into consideration the dual antithetical relationships of sperm count and hyaluronidase content. The quantitative effects of these two variables upon fertilization are given by the multiple regression equation coefficients. Any attempt to establish optimal and minimal sperm concentrations for fertilization, therefore, should be referable to a constant minimal value of hyaluronidase. Similarly, the establishment of a threshold amount of hyaluronidase for fertilization should be based on a constant number of spermatozoa. For insemination purposes, the ideal semen specimen should contain a maximum number of spermatozoa and the smallest hyaluronidase content still capable of preparing the ovum for fertilization.

Since dilution of a semen specimen does not alter the ratio of hyaluronidase to sperm numbers, there will be no significant change in the percentage fertilization obtained with dilution up to a point. However, when dilution becomes great enough so that the sperm count falls below the threshold number for the amount of hyaluronidase present or, conversely, when the hyaluronidase titer becomes inadequate for fertilization, percentage fertilization will be materially affected. In this regard Salisbury (14) found that, in the insemination of 5765 cattle, dilutions of 1:40, 1:60, 1:80 and 1:100 of the same semen specimen gave no significant differences in percentage fertilization. Equation V may be used for predicting percentage fertilization in cattle. However, for use with highly diluted semen it must first be ascertained whether sperm count and/or hyaluronidase titer fall below threshold values required for the process of fertilization.

In a few cases of human infertility characterized by oligospermia, it is possible that the addition of hyaluronidase might result in conception. In many more instances of infertility where the semen is incriminated, however, it is likely that too great an amount of hyaluronidase is already present in relation to the number of spermatozoa. In cases such as these, the development of methods for reducing the hyaluronidase content without affecting the spermatozoa present may well lead to an increased conception rate.

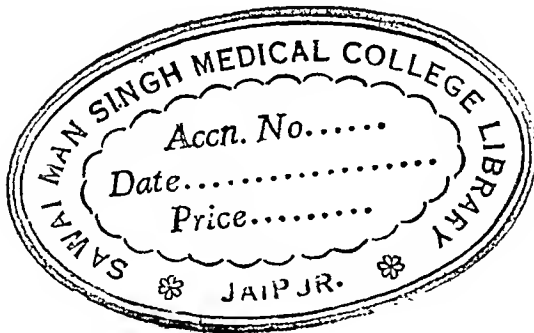
SUMMARY

1. Spermatozoa count was found to be significantly correlated with hyaluronidase titer and with percentage fertilization in cattle.

2. Hyaluronidase titer, beyond a threshold amount, has been found to have a relatively high negative correlation with percentage fertilization.
3. A multiple regression equation for percentage fertilization, with sperm count and hyaluronidase titer as the two independent variables, has been derived.
4. Criteria for the establishment of minimal and optimal sperm concentrations and of an ideal seminal specimen for fertilization have been set forth.
5. The use of a predictive equation for percentage fertilization in the insemination of cattle, along with the probable effect of semen dilution, is suggested.
6. The relation of hyaluronidase to the possible therapeutic treatment of certain types of human male infertility was indicated.

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EFFECTS OF CERTAIN FACTORS UPON THE LEVEL OF THE PLASMA PHOSPHATASES OF BREEDING BULLS¹

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Received for publication October 24, 1947

Though numerous studies have related the plasma alkaline phosphatase level of human beings and experimental animals to the metabolism of calcium and phosphorus, many recent investigations show that variations in the phosphatase level of plasma and other tissues may be accompanied or caused by a number of different physiological and pathological conditions.

The results of a limited number of investigations of the plasma phosphatases of the bovine have been variable. Allcroft and Folley (1) reported a wide range of serum phosphatase concentrations in cows with the activity somewhat increased during pregnancy, whereas Auchinachie and Emslie (2) found the opposite trend. Although no relationship was found between the milking capacity and the enzyme concentration, the enzyme level decreased with advancing age until maturity was reached (1). Wilson and Hart (3) demonstrated a high plasma phosphatase level three weeks or more before calving with a decrease near the time of parturition followed by a tendency to increase. A similar general observation was reported by Hibbs *et al.* (4). Phillips (5) demonstrated that the plasma phosphatase of cattle suffering from fluorine toxicosis increased in proportion with the fluorine intake and indicated that the enzyme offers a sensitive test for this disturbance. The plasma calcium and phosphorus remained within normal limits. No data are reported on the plasma phosphatase of bulls.

The object of this investigation was to study the relationships of diet, semen production, plasma calcium and phosphorus and age to the plasma phosphatases of dairy bulls.

EXPERIMENTAL

Twenty-three bulls from which semen was obtained at varying rates were employed in this study. Eleven of these animals constituted two groups in a nutrition experiment in which six bulls received a complex concentrate mixture containing supplemental minerals and vitamins and five bulls were fed a simple concentrate mixture consisting largely of corn with no mineral or vitamin supplementation. These animals received the same average grade mixed hay at the rate of 1 lb. per 100 lb. body weight. The concentrate mixtures were fed in sufficient quantity with hay to supply 1.18 lb. of digestible nutrients per 100 lb. of body weight daily. No more than three weeks difference existed in the age of the 11 bulls used in this phase of the study. These bulls were 27 months old at the beginning and 33 months old at the termination of the experiment.

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The remaining 12 bulls ranging from 17 to 128 months of age were employed in an artificial breeding stud and were fed in a manner considered to be consistent with the production of high quality semen.

The activity of the alkaline and acid phosphatases was determined in plasma at pH 9.3 and 5.0, respectively, by the method of King and Armstrong (6) as modified by Wiese *et al.* (7). A unit of phosphatase was defined as one mgm. phenol liberated from the disodium phenyl phosphate substrate in 30 minutes at 37°C. by 100 ml. plasma.

To facilitate quantitative expression, the average daily number of ejaculates, volume of semen and number of spermatozoa were calculated from the total number of ejaculates and spermatozoa and volume of semen obtained during the arbitrarily selected period of three months, terminating with the estimation of the plasma phosphatases.

TABLE 1. EFFECT OF REST UPON THE PLASMA ALKALINE PHOSPHATASE LEVEL OF BREEDING BULLS

BULL NO	DURING HEAVY USE	AFTER 52-DAY REST	INCREASE
	<i>units</i>	<i>units</i>	<i>units</i>
2	6.88	11.60	4.72
3	3.37	10.75	7.38
4	2.27	9.31	7.04
11	5.27	10.84	5.57
13	5.03	10.59	5.56
6	7.13	13.07	5.94
9	4.43	10.58	6.15
10	3.50	10.03	6.53
12	4.80	10.51	5.71
14	6.60	12.14	5.54
Average.....	4.93	10.95	6.02

RESULTS

Effect of diet on plasma phosphatase level. Since numerous reports indicate a possible relationship between the nutrition of animals and the alkaline phosphatase activity of their plasma, the concentration was measured in the plasma of two groups of bulls receiving extremely different concentrate mixtures.

Since these rations did not appear to affect the acid or alkaline phosphatase concentration of the plasma of these bulls, individual group data for phosphatase levels and data relative to the composition of diet are not given.

Effect of rest on plasma phosphatases of breeding bulls. It was observed that the plasma of bulls used for semen production sparingly or not at all contained higher concentrations of alkaline phosphatase than bulls ejaculated more frequently.

Table 1 shows a 2.2-fold increase in the alkaline phosphatase upon resting (for 52 days) bulls which had been yielding an ejaculate at 3 to 5-day intervals. The act of resting bulls from semen collection had no perceptible effect on the activity of the plasma acid phosphatase.

Relationship of rate and quantity of semen ejaculation to plasma phosphatases. An examination was made of the relationship of the frequency with which semen was obtained, the volume of semen and concentration of spermatozoa produced to the plasma phosphatases. The quantitative expression of the average daily figures used in these relationships appeared to be justified since semen was obtained at fairly regular intervals regardless of the rate.

Table 2 demonstrates the effect of taking semen on the plasma alkaline phosphatase of a bull from which semen had not previously been obtained. The collection of nine samples of semen totaling 193 ml., obtained by massaging the ampullae of the ductus deferens and seminal vesicles during a 16-day period, effected an abrupt decline in enzyme concentration from 18.95 (range 18.04 to 19.75 in five samplings) to 5.24 units per 100 ml. plasma.

TABLE 2. DECLINE OF PLASMA ALKALINE PHOSPHATASE LEVEL IN A BULL FROM WHICH SEMEN HAD NEVER BEFORE BEEN OBTAINED

PHOSPHATASE BEFORE SEMEN COLLECTION	SEMEN COLLECTION PERIOD	SAMPLES OBTAINED	SEMEN VOLUME	SPERMATOOZA NO.	PHOSPHATASE AFTER SEMEN COLLECTION
<i>units</i>	<i>days</i>	<i>no.</i>	<i>ml.</i>	<i>billions</i>	<i>units</i>
18.95 (18.04-19.75) ¹	16	9	193	4.14	5.24

¹ Range found in five samplings during a 6-month period.

TABLE 3. EFFECT OF FREQUENCY AND QUANTITY OF SEMEN EJACULATION UPON PLASMA ALKALINE PHOSPHATASE LEVEL

	PHOSPHATASE RANGE (UNITS)				CORRELATION COEFFICIENT AND STANDARD ERROR
	2.00-5.99	6.00-9.99	10.00-13.99	14.00-19.75	
Ejaculates/day.....	.26	.28	.10	.01	-0.71 ±0.06
Volume/day (ml.).....	1.28	1.04	.43	.05	-0.83 ±0.04
Spermatozoa/day × 10 ⁸	13.26	11.11	4.32	.57	-0.86 ±0.03
No. cases.....	13	21	25	6	

A marked decline in plasma alkaline phosphatase concentration was found to accompany an increase in the ejaculation rate and in the volume of semen and number of spermatozoa ejaculated. The relationships are established in table 3 and figure 1. The degree of relationship of the plasma alkaline phosphatase level was highest with the number of spermatozoa ejaculated, intermediate with the volume of semen ejaculated and lowest with the number of ejaculates yielded. All of these were highly significant statistically.

The concentration of plasma acid phosphatase did not appear to be related to the rate or quantity of semen ejaculated.

Relationship of plasma calcium and phosphorus and age to plasma phosphatases. The extensive use of plasma alkaline phosphatase values in the detection of disturbed bone metabolism and the decline accompanying aging, found by a number

of workers, made it necessary to establish whether or not the observed differences in enzyme concentration of bulls' plasma were associated with the levels of plasma calcium and phosphorus or the age of bulls. It was found that the calcium and phosphorus concentrations in the plasma of these animals were not greatly different regardless of the alkaline phosphatase activity and that age was without appreciable effect upon the enzyme level.

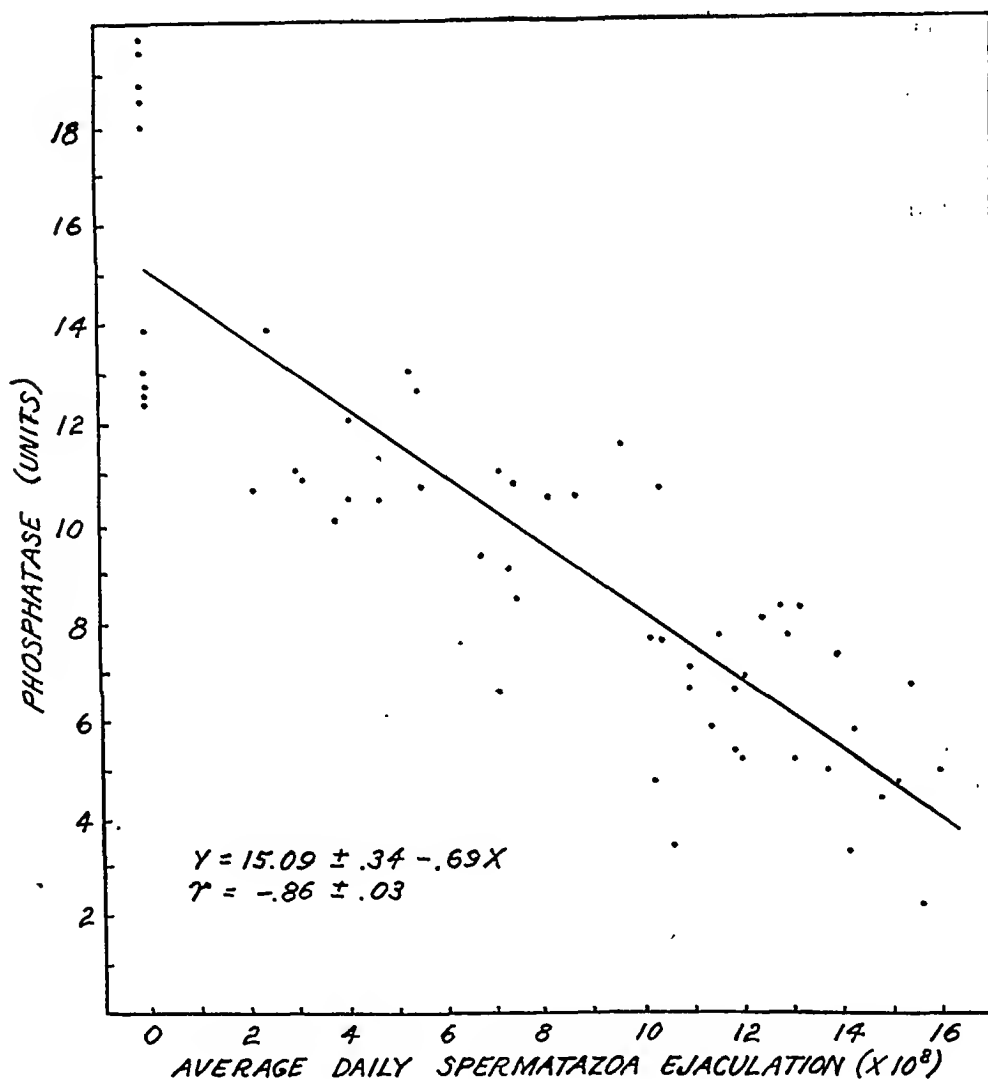


FIG. 1

The acid phosphatase concentration did not bear any noticeable relationship to the plasma levels of calcium and inorganic phosphorus or age.

DISCUSSION

Although dietary influences on the plasma alkaline phosphatase are widely known, the limits of the rations fed in this experiment were without effect upon

the activity of the enzyme in the plasma of bulls. The complex concentrate feed used in this study provided 3.62 grams more Ca, 1.91 grams more P, 40 mgm. more Fe, 73 mgm. more Mn, 6 mgm. more Cu, 0.45 mgm. more Co, 37.10 grams more protein, 11.16 grams more fat, 13,620 I. U. more vitamin A and 2,043 U. S. P. units more vitamin D per pound than was supplied in the same amount of the simple concentrate feed. The literature demonstrates the effects of certain of these dietary factors upon the phosphatase level of plasma and other tissues of humans and experimental animals; however, in view of the great differences of the two rations employed in this investigation, the possibility of demonstrating a dietary influence on the concentration of plasma phosphatases of breeding bulls seems remote.

Since it is known that cows in widely different mineral balances seem to maintain similar plasma levels of calcium and phosphorus, these data involving plasma calcium and phosphorus should not be interpreted to mean that the mineral metabolism of the bull is not related to alkaline phosphatase.

The observation of a declining phosphatase concentration with aging in experimental animals (1) and humans (8) may be a secondary reflection of bone metabolism. The small number of very young and old bulls available for study limited the age range used in this investigation; however, it was found that the plasma of new-born bull calves contained much greater quantities of the alkaline phosphatase than did that of older bulls (9).

Although the plasma alkaline phosphatase decreases in a consistent manner with increased rates of semen ejaculation and quantities of semen yielded, these data do not allow the mechanism involved in the decline of the enzyme in plasma to be ascertained. It seems indicated, though, that the semen alkaline phosphatase may be derived from the blood at which expense the enzyme level may be maintained in the semen. The work of Gutman and Gutman (10) shows that the concentration of alkaline phosphatase in the seminal fluids of man is very low and, in this respect, is opposite to acid phosphatase. The results of a few determinations conducted in this laboratory would indicate a very high concentration of alkaline phosphatase in bull semen (9). A study of the enzyme in bull semen will be published later.

It is not known what practical husbandry application these findings may have; however, if a sufficiently large number of bulls were examined, it appears probable that these data could be used as an index of when a rest from the breeding routine might be beneficial to bulls.

SUMMARY

1. The plasma alkaline phosphatase concentration of bulls appears to be largely dependent upon the frequency of semen ejaculation and, more particularly, upon the number of spermatozoa yielded.

2. The rations used in this investigation did not elicit any perceptible influence on the plasma phosphatases of bulls.

3. No relationship was observed between the plasma level of alkaline or acid phosphatase and the plasma levels of calcium, inorganic phosphorus or age of bulls.

4. The level of acid phosphatase was not affected by the rate of semen collection or quantity of semen ejaculated.

5. In 144 analyses made on the plasma of bulls, the average alkaline phosphatase activity was 8.98 (range 1.99–21.03) units per 100 ml. and the average acid phosphatase was 2.09 (range 0.35–6.63) units per 100 ml.

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UTILIZATION BY DOGS OF THE NITROGEN OF HEATED CASEIN

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Received for publication November 3, 1947

Earlier investigations have shown that heat treatment of proteins with temperatures over 120° appreciably lowers their nutritive value as determined by rat growth and balance methods (1-4). Morgan, Greaves and Loveen (5) reported that the growth value of casein for rats was decreased 17 per cent when heated at 140° C. for 30 minutes, but that its digestibility was lowered only 4 per cent. They concluded that lysine was the first amino acid damaged and histidine the second when casein was heated to this temperature. Arnold and Elvehjem (6) observed running fits in dogs fed a heated diet of wheat and meat scraps and noted some improvement when lysine was given. No other work with dogs fed heated proteins has been reported. This study was undertaken largely to determine whether the response of rats to heated diets can be duplicated in another species.

Two litters of mongrel dogs and two litters of purebred cocker spaniels, a total of 17 dogs, were used in this experiment. Some of each group were placed on a diet containing unheated casein at the same level used for the littermates fed the diet containing heated casein. The effect of these regimes was noted on the growth, blood composition, nitrogen utilization and tissue changes.

METHODS

The same type of casein was used in all the experiments, an acid-precipitated, dried commercial product designated raw casein, and the same heated for 15 or 30 minutes in layers one to one and one-half inches deep at 130°, 140° or 200°. The temperature of the casein was carefully controlled by preheating the oven to a higher temperature than that desired so that the introduction of the amount of casein prepared at one time brought the material to the desired temperature within 10 minutes. The oven temperature was then controlled so that the casein remained at the desired temperature for either 15 or 30 minutes. In this way all lots of casein prepared for a given experiment were subjected to identical treatment. The casein heated at 130° and 140° was light yellowish buff in color and that heated at 200° a considerably darker shade. The depth of color was found to vary quite consistently with the temperature to which the casein was exposed.

The animals assigned to the heated diet were started on a casein heated at 130°. Since this did not seem to slow their growth measurably, the temperature of heating was increased to 200° after 46 days. This diet produced such severely deleterious effects that in the later experiments a lower temperature, 140°, was

used. The diets were made up of casein, raw or heated, 18, 24 or 36, CaCO_3 1.5, hydrogenated cottonseed oil 10.0, salt mixture #5,¹ 2.4, cornstarch and sucrose in equal amounts to make the total, 100. In addition each dog received one gram yeast and 1 gram wheat germ per kgm. per day except preceding and during periods of collection of excreta, when they were fed the crystalline vitamins, per kgm. per day, thiamine, pyridoxine, and riboflavin each 0.1 mgm., pantothenic and nicotinic acid each 2 mgm. Grayfish oil fortified by irradiated ergosterol was fed to supply vitamins A and D in the amount of 400 I.U. of vitamin A and 50 I.U. of vitamin D per kgm. per day. The protein supplied by the yeast and wheat germ amounted to 7 to 18 per cent of the total protein fed, the proportion increasing with the lowering of intake in the older dogs.

The dogs were kept in individual standard metabolism cages. From the time they were weaned at five to seven weeks of age until they were four months old, they were given the weighed daily ration in two feedings. After they were four months old, the ration was given in one feeding daily. The dogs on the unheated diet ate their food promptly and completely, as did usually also those on the heated diet. The animals in each group were paired, one of the pair being placed on the raw and the other on the heated diet, and each two animals thus paired were fed daily the same amount of nitrogen and total food, regardless of differences in weight increases. The amount of food given decreased from 200 cal/kgm/day at first to 80 cal/kgm/day when the dogs' growth rate levelled off after seven to eight months.

Total serum protein and serum albumin were determined by the micro-Kjeldahl method, and nonprotein nitrogen was similarly measured on the Folin-Wu filtrate. Serum albumin and globulin were fractionated by the method of Kingsley (7) except in some of the earliest determinations in which Howe's method (8) was used. Simultaneous determinations by the two methods indicated that comparable results could be obtained. Because of its rapidity, Kingsley's method was chosen.

The nitrogen content of pooled five-day urine and fecal collections was measured by macro-Kjeldahl determinations. The urine was preserved with a small amount of acid and toluene and the feces were dried daily. Diarrhea, particularly on the heated diets, sometimes interfered with the fecal collections, and in these instances less than five-day collections were used.

Creatine was determined in the muscle tissue of the dogs of group II by the method of Rose, Helmer and Chanutin (9). Vitamin A was determined in the livers of this group by the method of Davies (10) and in the serum by the method of Yudkin (11).

EXPERIMENTAL RESULTS

Growth. As shown by the growth curves (fig. 1, 2 and 3), the dogs fed the heated diet made poorer growth than the littermates fed the same amounts of the raw diet with only one exception, *dog 13* of group III, which was heavier than

¹ Axelrod, H. E., A. F. Morgan and S. Lepkovsky. J. Biol. Chem., 160: 155, 1945.

his control *dog 14*, at the start. The stunting was corrected by rapid compensatory growth whenever an animal was changed from the heated to the raw diet, as was done in the case of *dog 8*, and the reverse condition occurred when the heated was substituted for the raw diet as in the cases of *dogs 9* and *3*.

There was less severe depression of growth in the group (III) fed the casein heated at 140° than in the two groups fed the more highly heated diet. Three of the former group were given the diet containing 24 per cent casein and four

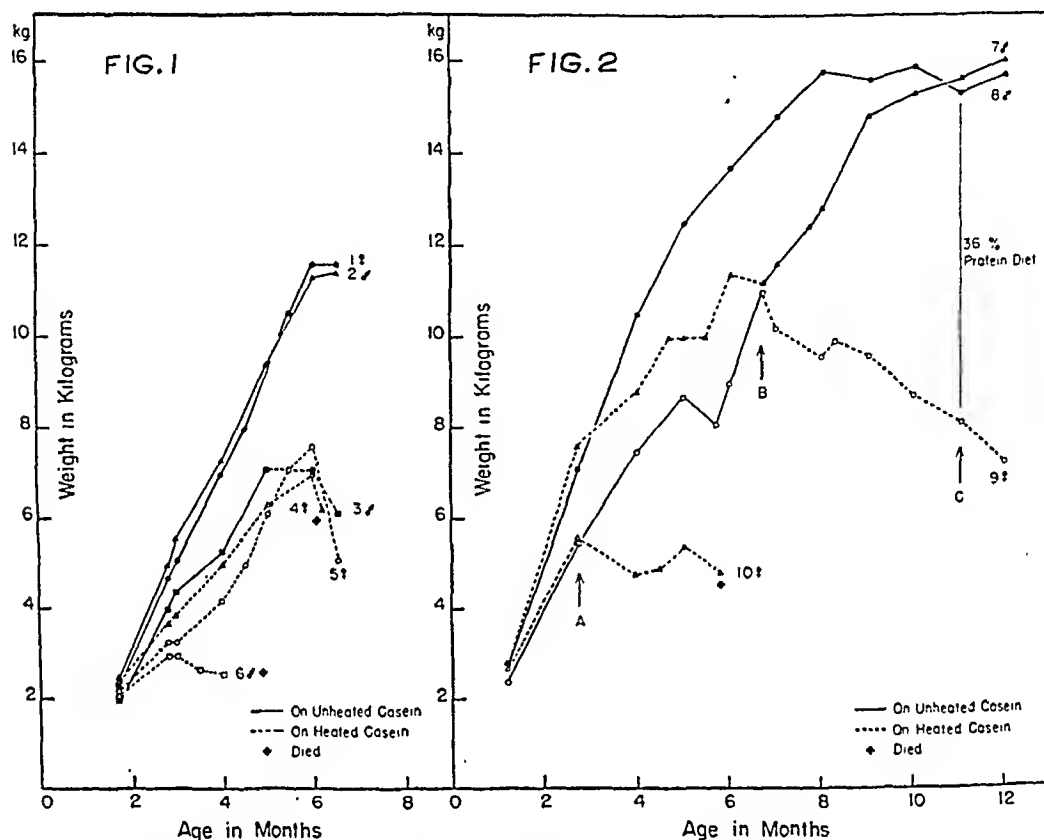


FIG. 1. GROWTH OF GROUP I, hounds, fed from weaning purified diets containing 18 per cent casein, unheated or heated 15 minutes at 200° .

FIG. 2. GROWTH OF GROUP II, shepherd dogs, fed from weaning on purified diets containing 18 per cent casein, unheated or heated. At first the casein was heated 30 minutes at 130° , but at point A was changed to casein heated 15 minutes at 200° . At B, *dog 7* was changed from heated to unheated, and *dog 9* from unheated to heated diet. At C, the level of casein in both diets was raised to 36 per cent.

received an 18 per cent casein diet. Two of those fed the 24 per cent diet, *dogs 16* and *17*, were of a different litter from the other five, and grew less rapidly, but the difference between the dogs on the raw and heated diets remained the same. When *dog 9* of group II was changed from a diet containing 18 per cent heated casein to one containing 36 per cent heated casein, her condition rapidly deteriorated.

Three measures were tried in an attempt to improve the growth of the dogs on

the heated diets: 1) the addition of L-lysine dihydrochloride² to the experimental diet, 2) the similar addition of lactalbumin, a commercial product and 3) the substitution for the experimental diets of the stock diet. Dogs 5, 6 and 17 were

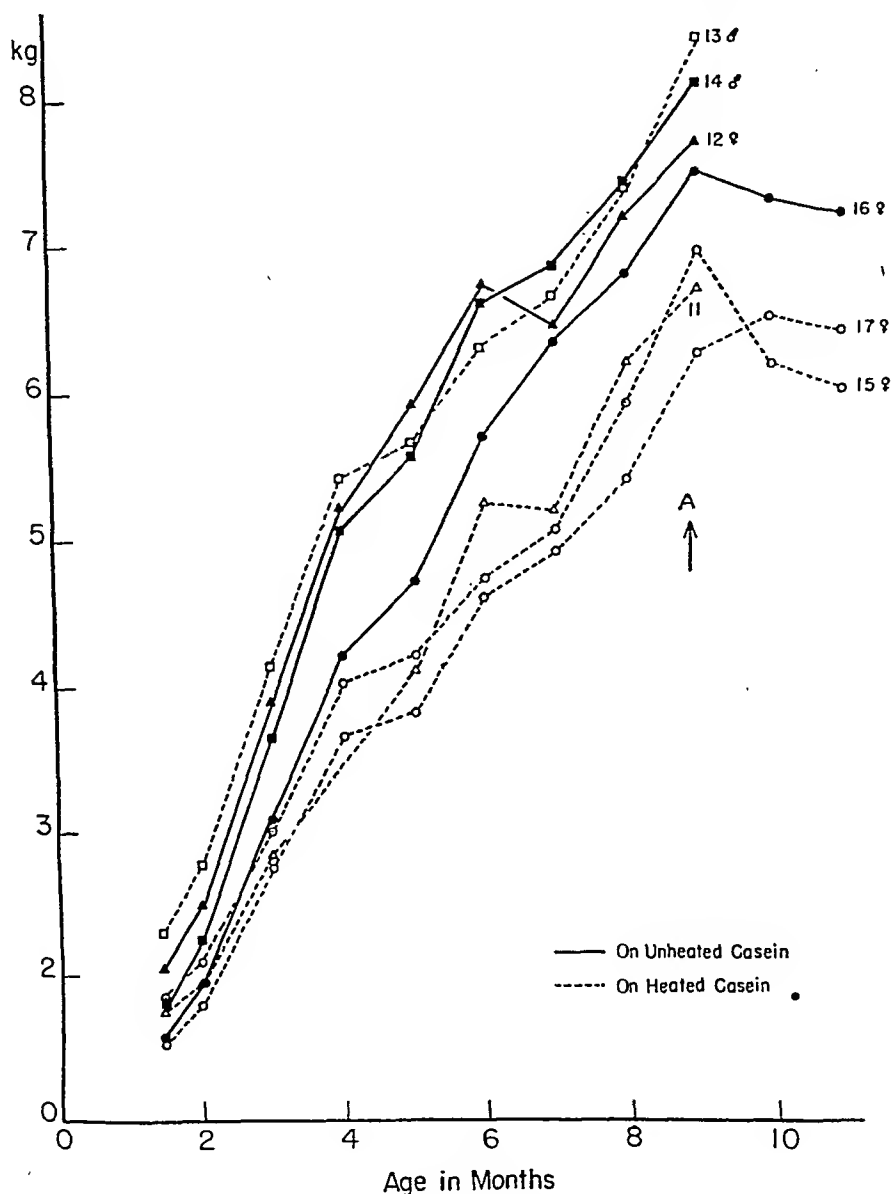


FIG. 3. GROWTH OF GROUP III, cocker spaniels, fed from weaning on purified diets containing 18 or 24 per cent casein, unheated or heated 15 minutes at 140°. Dogs 15, 16 and 17 received the 24 per cent diet, the others, 18 per cent. At A, force feeding of 15 and 17 was discontinued.

given the lysine; dog 6 after 80 days by subcutaneous injection, 0.5 gram daily for 10 days; dog 5 after 135 days, 1 gram daily by mouth for 10 days; and dog 17

² The lysine was generously supplied by Merck & Co., Rahway, New Jersey.

after 135 days, 2 grams daily for eight days by mouth, and, 23 days later when more lysine became available, 1 gram daily for 20 days. The amounts given in the cases of *dogs 5* and *6* were apparently too small to be of advantage, but *dog 17* made some improvement in growth, in serum protein levels and in nitrogen retention during, but more markedly in the period after, the lysine administration. The nitrogen loss by *dog 5* was also decreased somewhat in the period following the lysine feeding.

Dogs 13 and *14* of group III after five months were each given in addition to their usual diet 3 grams dry laetalbumin daily, amounting to 60 mgm. nitrogen per kilo per day added to the 213 to 220 mgm. per kilo per day contained in their basal diet. During the two months that this supplement was given the growth, nitrogen retention and serum protein level of *dog 13* on heated diet were improved, but there appeared to be no change in *dog 14* on the unheated diet.

All seven of the dogs of group III were placed on the 36 per cent casein stock diet after 7 to 13 months on the experimental diets. All the dogs which had received the heated diets at once improved in weight, condition of coat, activity and appetite.

The animals in group III which received the less severely heat-treated casein had few signs of inferiority other than slow growth, although their hair was dry and short as compared with their controls receiving the unheated diet. In groups I and II, however, the dogs on the diet containing casein heated at 200° had scaly dry skin, dull short hair and recurring skin lesions, accompanied by loss of hair, particularly about the eyes. Two dogs in group I and one in group II died during the experiment, apparently because of the deficiency.

Serum proteins. In all cases the levels of total serum proteins and serum albumin of the dogs fed the heated casein were lower than those of the corresponding animals on the unheated protein. There were larger differences shown by the groups fed the more severely heated diets, as illustrated in figure 4. The level of circulating serum proteins and albumin found after three to five months on the diets in groups I and II showed depression of both values in *dogs 5*, *4* and *9* as compared with *dogs 1*, *2* and *8*, their pair-fed controls on the unheated protein. The changes in these levels produced in two months in *dog 3* when removed from the unheated to the heated diet and in three months in *dog 7* when changed from the heated to the unheated diet were striking and consistent. In figure 5 is shown the decrease in the serum protein level of *dog 9* which followed the change in the level of heated protein in the diet from 18 per cent to 36 per cent, and the fall in all these serum values when the three dogs of group II were placed on low-nitrogen diets approximating the endogenous level.

The dogs of group III which received the less severely heated diet also had total serum proteins and albumin levels slightly lower than had their controls on the unheated diets. The group fed the 18 per cent casein diets had about the same serum protein levels as those fed the 24 per cent casein diets (fig. 6), and the differences between those on the heated and unheated protein appeared to be of the same order. However, *dogs 15* and *17* fed a heated 24 per cent diet maintained somewhat higher serum albumin than *dogs 11* and *13* fed the heated 18 per cent

diet. When *dogs 13* and *14* were each given three grams lactalbumin per day, a rise in serum protein level occurred in *dog 13*, but little change in albumin level. When all four dogs of this group were placed on the 36 per cent unheated casein stock diet, in two to three months all had attained the same serum protein and albumin levels. When 1-lysine dihydrochloride was given *dog 17*, 40 grams in 52 days, the serum protein and albumin levels rose perceptibly above those of the pair-fed *dog 15* on unsupplemented similar diet but did not equal the levels shown

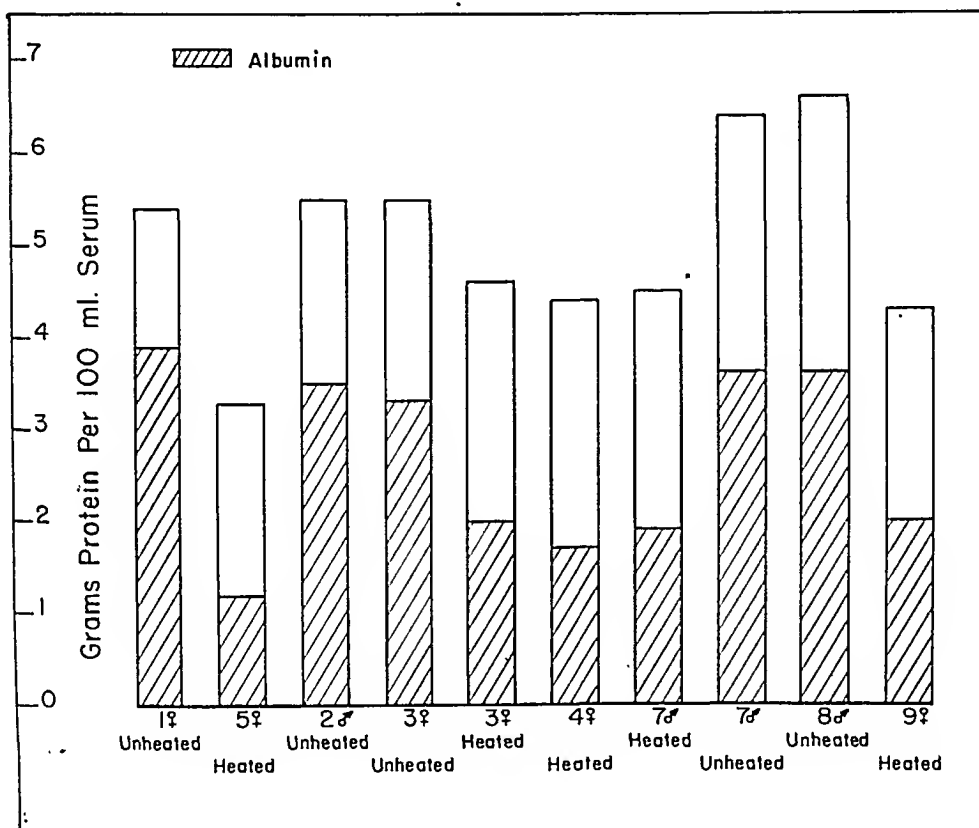


FIG. 4. SERUM PROTEIN AND ALBUMIN AS AFFECTED BY HEATING OF DIETARY CASEIN. The number of the dog is given below the columns. These columns represent the blood protein values after two to five months on the diets. *Dogs 2* and *8* were on unheated diet throughout, *dogs 5* and *4* on heated (200° for 15 minutes) casein throughout. *Dog 3* was first on unheated diet for three to four months, then heated diet for two months. *Dog 7* was on heated diet for six months then unheated diet for six months. *Dog 9* had been on heated diet for three months at this time.

by the animal, *dog 16*, given the unheated protein. These three dogs were maintained on the experimental diets several months longer than any of the others, and it was noted that after maturity was reached, at 10 to 12 months of age, the differences in serum protein levels tended to decrease.

Hemoglobin. From the fourth to the eighth month on the diet the dogs of group III on heated diet had 11.6 to 14.9 grams hemoglobin per 100 ml. blood and those on the unheated diet had 13.2 to 15.8. The per cent of packed cells

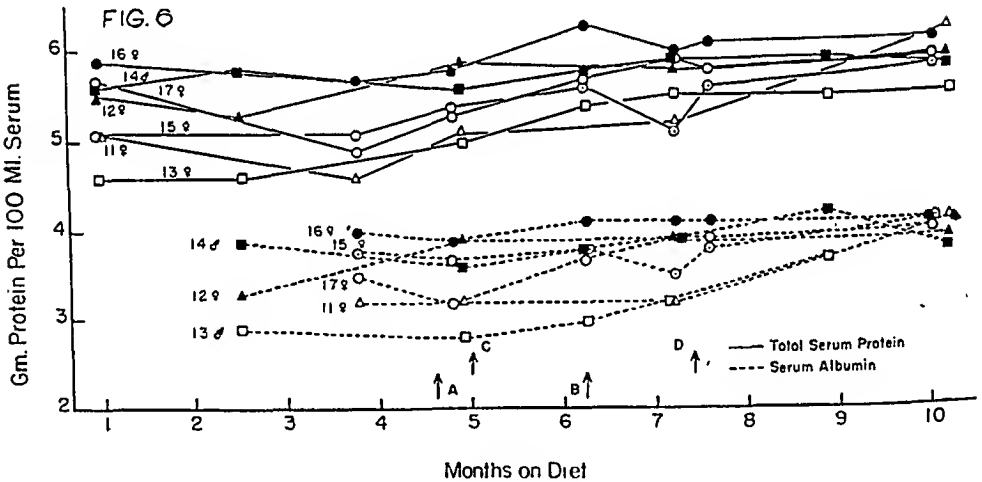
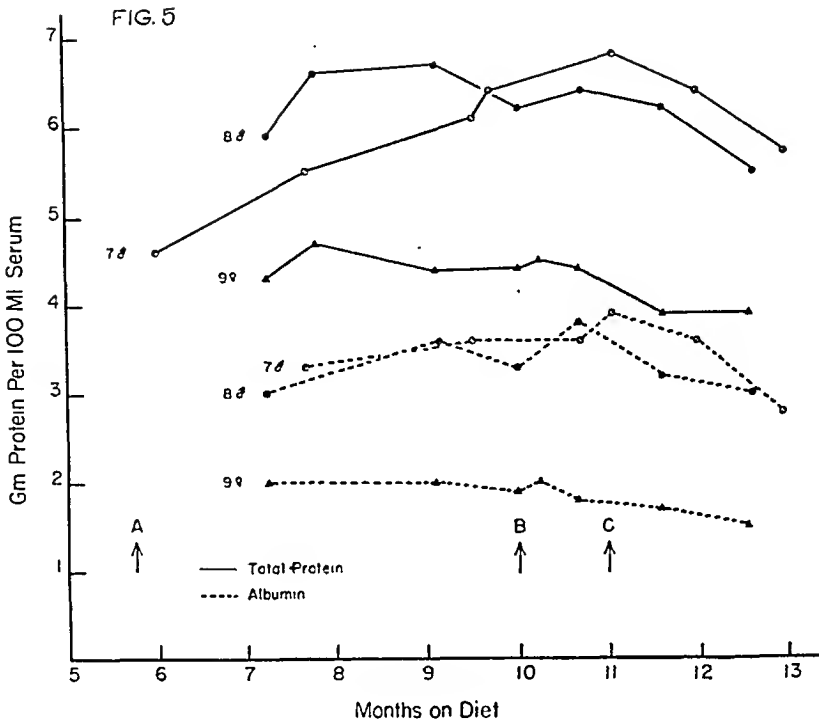


FIG. 5. SERUM PROTEIN AND ALBUMIN LEVELS OF GROUP II. At A, dog 7 was changed from 18 per cent heated casein (200° for 15 minutes) to the unheated diet, and dog 9 from unheated to heated diet. At B, dogs 7 and 8 were changed to unheated casein, 36 per cent, and dog 9 to heated casein, 36 per cent. At C all three were placed on the nonprotein diet, followed by intakes at the endogenous level.

FIG. 6. SERUM PROTEIN AND ALBUMIN LEVELS OF GROUP III. At C began 3 grams lactalbumin daily administration to dogs 13 and 14, continued to D. Between A and B, 40 grams dl-lysine hydrochloride was given dog 17. At D, dogs 11, 12, 13 and 14 were changed to 36 per cent unheated casein diet.

was also usually larger in the latter. The dogs of groups I and II which were fed the severely heated casein after three to five months on the diet had only 8.4 to 9.6 grams hemoglobin per 100 ml. blood while their pair-fed controls on the unheated diet had 11.7 to 14.7.

Nonprotein nitrogen. The level of blood nonprotein nitrogen varied very little throughout the feeding period. The dogs in all groups which were fed the unheated protein had 30 to 38 mgm. per cent and those fed the heated diet 17 to 28 mgm. per cent.

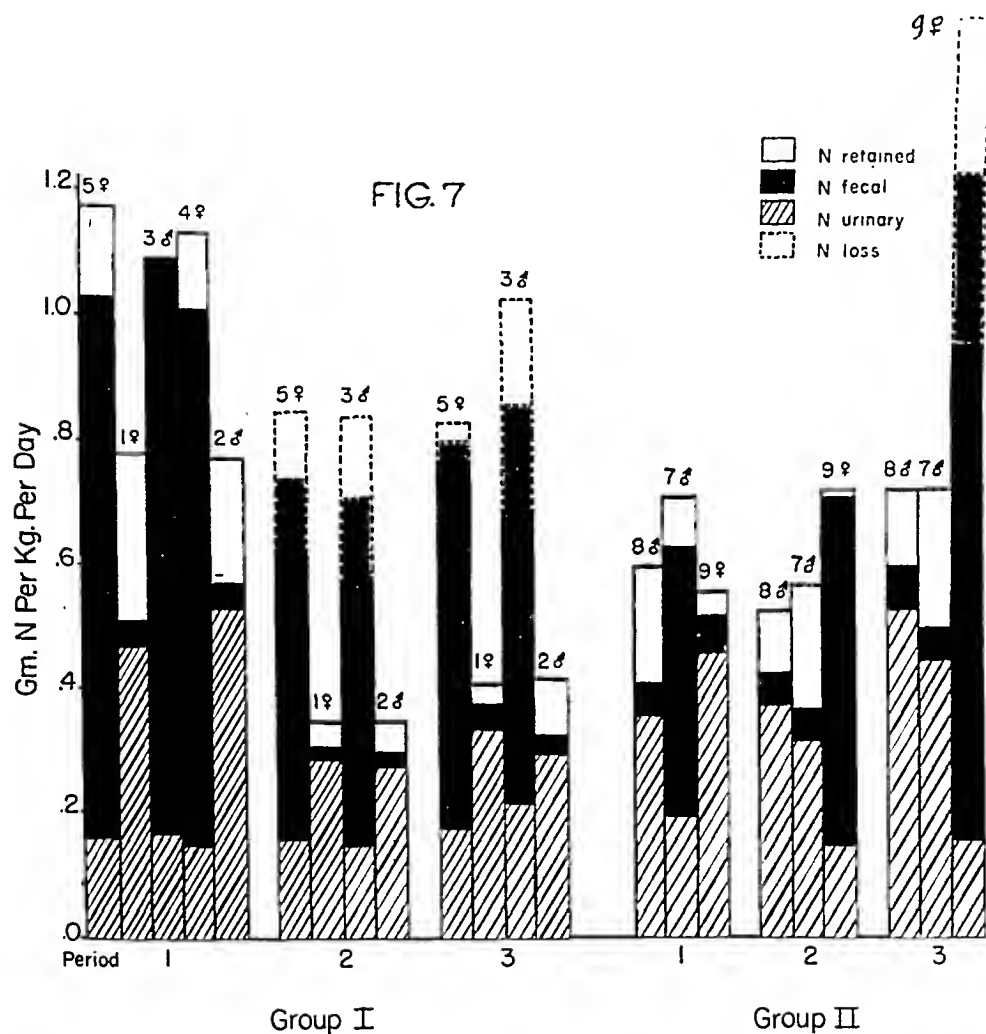


FIG. 7. NITROGEN BALANCES OF DOGS OF GROUPS I AND II. *Group I*, periods 1, 2 and 3, one balance each after 125, 139 and 146 days on the diet. *Group II*, period 1, two balances in the fourth month on the diets; period 2, three balances in the seventh and eighth months; period 3, one balance in the ninth month. Between periods 1 and 2, *dog 7* was shifted from heated to unheated diet and *dog 9* from unheated to heated diet.

Nitrogen balances. The intake of the dogs on the heated diet was kept parallel with that of their pairmates on the unheated protein during the balance periods by force feeding when necessary. These intakes were kept nearly equal regardless of differences in the weight of the animals. Each collection period was five days in length. Occasionally the dogs on heated diet excreted very soft feces, thus interrupting the collections.

As shown in figure 7 a large part of the nitrogen excreted appeared in the feces

of the dogs of group I fed the casein heated at 200°. In succeeding periods the nitrogen retention of these animals decreased and eventually became negative. The proportion of food-nitrogen lost in the feces increased from 44, 56 and 47 per cent in the first period, to 74, 84 and 76 in the second and 94 and 96 in the third. The two dogs on the unheated casein excreted in the feces only 5 to 10 per cent of the food nitrogen throughout the experiment. During the balance periods the latter dogs weighed 10 to 11 kgm., the former 5 to 7 kgm.

An attempt at determination of the endogenous level of nitrogen excretion was made with the three dogs of group II after they had been on the diet ten months. When fed nonprotein basal diet, after a five-day adjustment period, the urinary nitrogen excretion of the two dogs which had been fed the unheated casein was found to be similar, and their fecal output very small (table 1). The dog which had been subsisting on the heated diet excreted equal amounts of nitrogen in urine and feces. The pattern established on the heated protein regime apparently persisted. The dogs were then returned to their original casein diets but

TABLE 1. THE TRUE DIGESTIBILITY OF HEATED (200° FOR 15 MIN.) AND UNHEATED CASEIN
In mgm. per kgm. per day

DOG NO.	WEIGHT	PROTEIN FED	N INTAKE	URINE N	FECAL N	BALANCE N	ABSORBED N	TRUE DIGESTIBILITY
	kgm.							per cent
8♂	15.4	No protein	—	110	6	-116		
	13.0	Unheated casein	154	164	22	-32	138	89
7♂	15.7	No protein	—	113	11	-124		
	14.0	Unheated casein	143	145	30	-32	124	87
9♀	7.1	No protein	—	60	59	-119		
	5.9	Heated casein	170	52	178	-60	51	30

at approximately the endogenous level. The true digestibility of the unheated casein appeared to be 89 and 87 per cent and of the heated casein 30 per cent.

Earlier balance experiments with these three animals indicated large losses of nitrogen in the feces when the heated protein was fed, with greatly increased excretion when the level of casein was raised from 18 to 36 per cent (fig. 7, period 3). Dog 7, which had been depleted by the earlier heated diet regime, retained more nitrogen in every period on the unheated casein than did dog 8, which had been fed the unheated diet from the beginning.

All of the animals of group III fed the casein heated at 140° for 15 minutes (fig. 8) exhibited the same digestive pattern as did those in groups I and II, but the loss of nitrogen in the feces was less, usually 30 to 40 per cent of the total nitrogen excretion, even after seven months on the diet. None of the four dogs of this group which were fed the heated casein showed the severe failure of nutrition seen in the animals fed the more highly heated casein. Dog 17, which was given 1-lysine dihydrochloride in balance period 2 as previously mentioned, presented

better nitrogen retention than *dog 15* on heated casein without added lysine in this period only.

Dogs 11 and *13*, fed the 18 per cent heated casein diets, were force-fed in period 1 to conform with the intakes of their controls, *dogs 12* and *14*, but the intake of the latter was decreased to the voluntary intake of *dogs 11* and *13* in period 2. It will be seen that the nitrogen retention was much decreased. In period 3,

FIG. 8

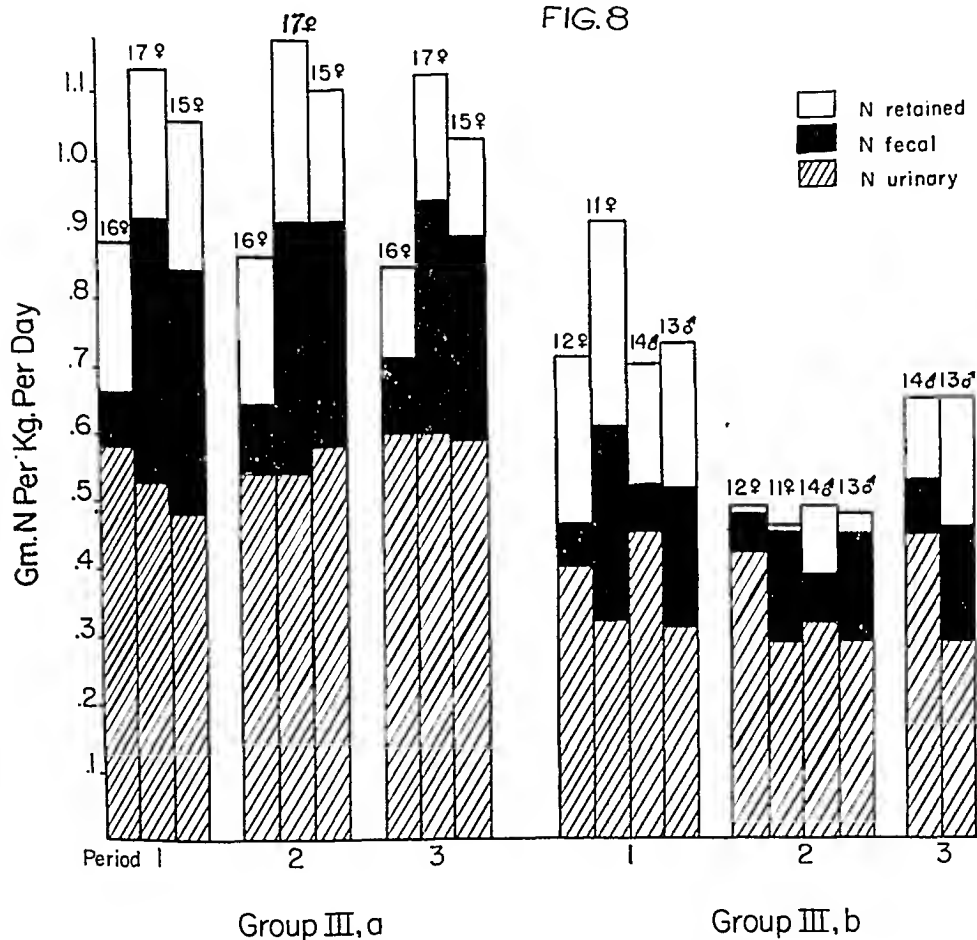


FIG. 8. NITROGEN BALANCES OF DOGS OF GROUP III. A. Dogs on 24 per cent casein diet. Period 1, six balances in the fourth and fifth months; period 2, three balances in the sixth month when 17 received one or more gram lysine daily; period 3, three balances in the seventh month. B. Dogs on 18 per cent casein diet. Period 1, two balances in the fourth month; period 2, one balance in the fifth month, no force feeding; period 3, three balances in the sixth month, when both dogs received 3 gram lactalbumin daily.

three grams lactalbumin was given daily to *dogs 13* and *14*, with accompanying improvement in the nitrogen retention of *dog 13*.

The retentions of these animals on heated diet during balance periods were often better than those of their controls on the unheated diet because of their greater intake in relation to body weight. The force-fed dogs sometimes made small gains in weight during the balance periods, but when the force feeding was

discontinued ceased to gain or lost weight. Their voluntary intake was seldom lower per kilogram weight, however, than that of the normal animals. This persistence of positive nitrogen balance with stationary or decreasing body weight has been noted by Frost and Risser (12) and by Robscheit-Robbins, Miller and Whipple (13).

The apparent digestibility of the more severely heated casein was 28 per cent, that of the less severely heated casein 67 per cent, and that of the unheated casein 91 per cent.

Running fits. No running fits occurred in the dogs fed the unheated or the uncomplicated heated diets. Two weeks after *dog 17* was given lysine as previously indicated, the animal had three running fits on three successive days. Thereafter fits of varying severity occurred at irregular intervals during the re-

TABLE 2. ANALYSIS OF FRESH TISSUES OF DOGS FED HEATED (200° FOR 15 MIN.) AND UNHEATED CASEIN

DOG NO.	DIET	CONSTITUENT	HEART	MUSCLE	LIVER	BLOOD PLASMA (AT AUTOPSY)
8♂	Unheated diet, 383 days.	Water, per cent	78.5	77.6	—	—
		Nitrogen, per cent	2.80	3.16	—	—
		Creatine, mgm. per cent	307	333	—	—
		Vitamin A, I.U. per gram	—	—	8640	1.08
7♂	Heated diet, 167 days, followed by unheated diet, 216 days.	Water, per cent	77.9	74.2	—	1.18
		Nitrogen, per cent	2.82	3.32	—	
		Creatine, mgm. per cent	342	367	—	
		Vitamin A, I.U. per gram	—	—	6960	
9♀	Unheated diet, 158 days, followed by heated diet, 215 days.	Water, per cent	78.7	76.4	—	0.35
		Nitrogen, per cent	2.69	3.32	—	
		Creatine, mgm. per cent	268	293	—	
		Vitamin A, I.U. per gram	—	—	8240	

maining six months of this animal's life on the heated diet. Three days after *dog 13* was placed on the stock diet containing 36 per cent unheated casein, the animal had a running fit. That an improvement in diet could precipitate this symptom seems unlikely, yet only these two animals out of 11 fed heated casein were seen to undergo this condition. *Dogs 7, 11 and 15* were also changed from heated to raw or stock diet without occurrence of fits. Interest is attached to this symptom because there is some evidence that severely heated dog foods of low or poor quality protein content may induce such fits (14).

Condition of tissues. Only the dogs in groups I and II were sacrificed. Exceedingly yellow livers in all the animals on heated diet were observed upon gross examination. The control livers were normal. Histological sections of the yellow livers made by staining with hematoxylin eosin showed vacuolization considered to be due to fatty infiltration, but stains for fat were not made. It is of

interest to note that *dog 7*, which was changed from heated to raw diet, had a normal liver, which would indicate that any damage which occurred in this organ was repairable.

The gastronemious and heart muscles of *dog 9* had low creatine values in both cases but normal total nitrogen contents, as compared with the creatine and nitrogen of the tissues of the two dogs of the same group which had been fed unheated diet. The decrease in creatine may be due to methionine deficiency in the heated casein.

The vitamin A of the liver of *dog 9* was of the same order as that of the two normal animals but that of the circulating blood was low (table 2). These determinations³ were made because *dog 9* had exhibited loss of hair, reddening and purulence of the eyelids, and skin eruptions which resembled the symptoms seen in vitamin A-deficient dogs. The utilization of vitamin A may have been impaired due to decrease in liver function, as suggested by Popper and Steigman (15), Ralli *et al.* (16) and others. This liver damage again may be referable to loss of lipotropic action in the heated casein due to changes in the methionine. Choline as such was not included in the diet but this was presumably supplied by the yeast and wheat germ used as the source of the B vitamins except during the actual nitrogen balance periods. The presence of normal vitamin A stores in the liver of *dog 9* with reduced plasma vitamin A level parallels conditions reported in clinical studies (17), and may be taken to mean that the damaged liver retained the vitamin in an inaccessible form or site (18).

DISCUSSION

The effect of heated casein on the metabolism of the dogs was shown by the decrease in rate of growth, the decrease in total serum protein and serum albumin, the decrease in nitrogen absorption, and the liver damage. Although true digestibility could not be determined in all cases, the large differences in apparent digestibility justify the conclusion that heating the protein seriously decreased its absorbability. It may also be concluded that the higher the temperature of heating of the casein, the greater the decrease in digestibility, since casein heated at 200° and fed at the 18 per cent level underwent a decrease in digestibility from 91 to 28 per cent, whereas that heated at 140° fell from 90 to 67 per cent. Little difference was apparent between digestibilities of the casein heated at 140° and fed at the 18 and 24 per cent levels, 68 and 66 per cents, respectively. To this lowered digestibility can probably be attributed most of the deleterious effects of the heated diet which are comparable with those of low protein diets.

It is well known that low protein diets may cause hepatic damage. Weech and Goettsch (19) who used a low protein diet of carrots and rice noted in dogs definite hepatic changes which they considered to be due to fatty infiltration. Elman and Heifetz (20) feeding the same type of carrot diet to dogs found extensive vacuolization of liver cells which was not due to accumulation of fat as shown by chemical analyses and stained sections, but which they ascribed to loss of liver

³ The vitamin A determinations were done by Lillian S. Bentley.

protein. This is parallel with observations made on some of the livers of dogs in this study which showed vacuolization which may have been caused by fatty infiltration, but since no fat stains or chemical analyses were made, the assumption may be unwarranted. Since the liver is considered to be of primary importance in the formation of plasma proteins (19, 21), this damage may be the cause of the fall in serum protein level. But quality and quantity of ingested protein also influence serum protein production (21). The series of serum protein determinations in groups II and III (fig. 5, 6) indicate that the serum protein level was directly and promptly influenced by nitrogen intake.

The results obtained with heated casein in these experiments are different from those previously described (1, 5) in which rats were used. In the rat, the digestibility of the heated casein was not markedly lowered, but the availability of the absorbed nitrogen was decreased. Supplementation of heated casein with lysine or histidine improved its biological value for rats. That the fraction of the heated casein nitrogen absorbed in these experiments with dogs was not as completely utilized as was that of the unheated casein can be inferred from the decrease in growth rate and lowering of serum protein levels which occurred in the animals fed large amounts of the heated protein. Thus the dogs in group III, which were fed heated casein at the 24 per cent level, might be expected to approximate the performance of their littermates fed the unheated casein at the 18 per cent level if the 34 per cent loss in digestion were the only disability inflicted. But neither growth nor serum protein levels bear out this conclusion (fig. 3 and 6). The serum protein and albumin levels shown by *dogs 15* and *17* were higher than those of *dogs 11* and *13*, but the positive control of the former dogs, *no. 16*, had also slightly higher levels than *dogs 12* and *14* which were fed the unheated diet containing 18 per cent casein. The globulin levels of all the dogs of this group fed the heated protein were about the same as those of their controls on the unheated diets, thus possibly indicating some failure of utilization of the absorbed heated nitrogen in albumin, but apparently not in globulin formation. This may mean that the amino acid fraction required for globulin formation was altered by heat more than was that required primarily for albumin formation. This is in agreement with the findings of Seeley (22), who reported that casein caused equal regeneration of albumin and globulin in depleted dogs, beef serum protein regenerated only albumin, but amigen, a casein hydrolysate, favored only globulin production. Seeley likewise found that depleted dogs excreted less urinary nitrogen than did normal animals, as was found to be the case in these experiments, all dogs on heated diets excreting less nitrogen in the urine, even on greater intakes and after allowance had been made for intestinal loss, than did those fed unheated protein (fig. 7, 8). The failure of casein acid hydrolysates fed intravenously to maintain weight and nitrogen balance in dogs, even when supplemented by cystine, as reported by Frost and Risser (12), may be in part at least due to the same type of heat damage manifested in whole casein. The fatty or vacuolized livers of their dogs, ascribed to sulfur amino-acid deficiency, were also seen in the dogs fed severely heated casein in these experiments.

The addition of 1-lysine dihydrochloride to the heated diets, which had been found remarkably effective in improving the growth of rats (1, 5), was also measurably useful in counteracting the heat effect in the one dog in which it was tried for a long enough period. The increase in growth, serum protein levels and nitrogen retention was unmistakable. There was, however, no accompanying alleviation of the digestive loss of nitrogen, and the animal did not equal its positive control, *dog 16*, nor greatly exceed the performance of *dog 15*, except in globulin formation, fed the heated casein without lysine supplement. It may be that the amount of lysine given was insufficient.

The improvement obtained with 3 grams lactalbumin daily in *dog 13* was similar. This much lactalbumin provided about 0.3 gram lysine, and 0.2 gram cystine and methionine combined, and the net effect equalled that of 1 gram lysine in *dog 17*. Since this simultaneous addition of lysine and methionine and cystine and other amino acids even in small amounts appeared to be as effective as the larger intake of lysine, the failure of absorption of the heated protein could hardly have been due entirely to unavailability of lysine.

The depression of biological value of a protein by heat may result from either decrease in absorbability of usable amino acids or in the availability of the completely absorbed amino acids. According to Seegers and Mattill (23) liver proteins heated at 120° for 72 hours or hot-alcohol-extracted for 130 hours had markedly lowered digestibility for rats, but acid hydrolysates of these proteins had the same digestibility and biological value as the original dried liver proteins. A later study (24) of the digestion *in vitro* of liver and kidney proteins, fresh and hot-alcohol-extracted, revealed the loss of digestibility in some of these proteins but not in others, and also a loss of free amino nitrogen without loss of total nitrogen. This was interpreted to indicate the formation in the denatured proteins of new enzyme-resistant anhydride linkages between the ϵ -amino group of lysine and available hydroxyl groups. Presumably these abnormal linkages were destroyed by complete acid hydrolysis.

In the case of the heated casein fed to dogs in our experiment the evidence points to failure of digestion and absorption as the primary and in the less severely heated protein possibly the entire cause of the decreased biological value. Apparently any enzyme-resistant peptides produced by dry heat are not absorbable when fed to the dog, as they are when fed to the rat, with resultant increase in fecal, and decrease in urinary, nitrogen in the former species. The net loss of biological value of the heated protein is nearly the same in the two species. Murlin, Nasset and Marsh (4) as a result of experiments with human subjects, reported reduction in egg-replacement value in wheat breakfast cereals which had been subjected to high temperatures, about 200°, in the process of manufacture. The reduction was of the same order as that reported for similar products fed to rats (1) and was ascribed to losses in metabolism with no reduction in true digestibility.

Mecham and Olcott (25) found that dry heat caused several proteins, including casein, to become increasingly insoluble and resistant to digestion *in vitro* by pancreatin. These effects were measurable in proteins heated at 110° to 140°

and much exaggerated when temperatures from 150° to 203° were used. This parallels the markedly increased loss of digestibility for dogs of the casein heated at 200° as compared with that heated at 140° in our experiments.

SUMMARY

1. Young dogs were fed from weaning to maturity on purified diets containing casein, unheated or heated at 140° or 200° for 15 or 30 minutes. The protein levels used were 18, 24 and 36 per cent of the diet.

2. The growth of the animals on the heated protein was inferior, particularly on that exposed to the higher temperature. Little advantage was seen in the use of the 24 per cent as compared with the 18 per cent heated diet and rapid deterioration occurred in one dog when 36 per cent heated casein was substituted for the 18 per cent diet.

3. The administration of 40 grams of 1-lysine dihydrochloride in 52 days to one dog caused slight but perceptible improvement in growth and serum protein level. A similar change occurred in another dog on the heated diet when 3 grams lactalbumin was given daily for two months.

4. The total serum protein and albumin levels, but not the globulin level, were progressively lowered in all the animals on the heated protein. Hemoglobin concentration was also decreased although less strikingly.

5. Total nitrogen intake was maintained equal in the paired animals on heated and unheated diets by force feeding. Nevertheless decreasing retention and eventual nitrogen loss occurred on the heated protein, with most of the excess excretion appearing in the feces. The apparent digestibility of the casein heated at 200° was 28, of that heated at 140°, 67, and of the unheated 91. True digestibility of the more severely heated casein in one case was found to be 30.

6. Running fits developed in the two dogs which had been treated with lysine or lactalbumin and in no others.

7. Yellow livers, apparently fatty, were found in all the dogs fed the 200° heated casein, and low heart and muscle creatine in one. These conditions point to possible methionine deficiency.

8. The differences between these observations and those seen previously in rats similarly fed indicate poorer capacity on the part of the dog for absorption of any abnormal amino acid or peptide forms present in the heated casein but similar net loss of biological value in the two species.

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OBSERVATIONS CONCERNING THE EFFECTS OF (1) SODIUM SALICYLATE AND (2) SODIUM SALICYLATE AND GLYCINE UPON THE PRODUCTION AND EXCRETION OF URIC ACID AND ALLANTOIN IN THE RAT¹

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Received for publication November 14, 1947

Salicylate is known to possess the property of increasing the urinary excretion of uric acid in both animal and man but the mechanism by which this is accomplished is not known.

Rockwood (1) believed that the drug acted to prevent the destruction of uric acid in the body, hence its increased excretion. Fine and Chace (2) however observed that concomitant with urinary increase in uric acid after the ingestion of salicylate, a decrease in blood uric acid occurred. Other workers (3, 6) have confirmed their findings, thus suggesting that the initial or primary site of salicylate action was probably renal.

Quick (7) believed that salicylic acid stimulated the renal excretion of uric acid. Gray and Grabfield (8) believed that denervation of the dog's kidney prevented the usual increase in urinary uric acid after the administration of salicylate. Talbott (9), employing various clearance techniques in his studies, was unable to find any change in the glomerular filtration rate of patients receiving salicylate, despite the fact that their urine did contain more uric acid. He concluded that salicylate probably interfered with the tubular reabsorption of uric acid. On the other hand, Klemperer and Bauer (10) found that the maximum urinary concentration of uric acid in subjects receiving salicylate was no different than that of control subjects. Because of this finding, they concluded that the increase in urinary concentration of uric acid following salicylate was not due to a change in renal tubular reabsorption.

In the present communication, results of studies concerning *a*) the renal hemodynamics and *b*) the uric acid and allantoin in the blood and urine of rats receiving sodium salicylate alone or combined with glycine are given. The evidence obtained strongly suggests that salicylate acts to increase the excretion of uric acid exactly as glycine was found to do (11), namely, by interfering with the renal tubular mechanism concerned with the excretion of uric acid.

METHODS

Both the acute and chronic experiments were done on male albino rats, weighing 275-350 grams, which were maintained on Purina Dog Chow.

¹ Aided by a grant from the Public Health Service.

Uric acid, creatinine and hippurate clearances were obtained on rats according to our previously described methods (11, 12).²

RESULTS

1. *Acute effect of sodium salicylate upon the uric acid output and upon the renal hemodynamics of the normal rat.* Twenty-three rats were used in this study. Uric acid, creatinine and hippurate clearances were obtained on 11 control rats and on 12 rats which received 10 mgm. of sodium salicylate in 10 cc. of H₂O per 100 grams of body weight by mouth at the beginning of the clearance study (10 A.M.) and 5 mgm. of sodium salicylate in 5 cc. of H₂O every hour during the collection period of five hours.

Sodium salicylate in the dosage used in these experiments increased the uric acid excretion promptly and markedly in the rat. Thus the average excretion of uric acid of the control rats was 0.147 mgm. per hour (see table 1 A) and that of the 12 rats receiving salicylate was 0.294 mgm. per hour (see table 1 B). This increase of approximately 100 per cent in the excretion of uric acid however was not preceded by any significant rise in the blood uric acid (compare table 1 A and B). Furthermore, this increased excretion of uric acid after salicylate was not accompanied by any significant change in the urine volume, rate of glomerular filtration or in rate of renal plasma flow (the latter two functions were measured by the creatinine and hippurate clearances respectively). The average control creatinine and hippurate clearances were 35.9 and 141.0 cc. per hour per 100 grams of body weight respectively (see table 1 A). After the administration of salicylate, these same clearances were 33.2 and 152.0 cc. respectively (see table 1 B).

2. *Acute effect of sodium salicylate upon the blood and urinary allantoin concentration of the normal rat.* Twelve rats were used. Control determinations of the allantoin excretion per hour over a two-hour period were made on six of these rats. Similar determinations were made on the remaining six rats except that each received the same quantity of salicylate as the rats described above.

In these experiments, the administration of salicylate was not found to have any effect either upon the blood allantoin or upon the amount of allantoin excreted in the urine. The average blood allantoin (1.63 mgm. per 100 cc.) of the

² In respect to our method of determining the hippurate clearance which recently was misunderstood by Friedman, *et al.* (13), it should be stressed that two blood samples taken at the beginning and end of the clearance period were analysed for hippurate. The average of these two values was then multiplied by a correction factor (0.715). The use of this factor was found (after preliminary determinations of the plasma concentration of hippurate) to correct for the changing rate of plasma hippurate decline occurring during the first and second hours of the clearance. It cannot be stressed too much that a hippurate clearance based upon a single plasma analysis is completely unreliable. Our rats received 100 mgm. of hippurate subcutaneously which represented a dosage of approximately 30-35 mgm. per 100 grams of weight. The plasma concentration of hippurate during the clearance after such a dose rarely exceeded 4 mgm. per 100 cc. Allantoin in the blood and urine of rats was determined according to the method of Christman, Fosterer and Esterer (14).

six control rats fell approximately 11 per cent during the clearance period, during which time the average amount of allantoin excreted was 2.16 mgm. per hour.

TABLE 1. ACUTE EFFECT OF SODIUM SALICYLATE UPON THE URIC ACID CONTENT OF THE NORMAL RAT'S BLOOD AND URINE, AND UPON ITS RENAL HEMODYNAMICS

RAT	BLOOD URIC ACID DURING CLEARANCE ¹			U.F.	C.C.	H.C.	U.A.	U.A.C.
	10:00	12:00	3:00					
A. Control rats								
52	1.20	1.50	1.50	1.3	38.6	212.0	0.156	14.8
72	0.95	1.00	1.20	1.2	39.4	139.0	0.163	15.1
54	0.92	1.40	1.20	1.3	37.6	149.0	0.158	12.4
73	1.40	1.40	1.50	0.8	34.6	139.0	0.163	—
42	0.81	0.95	0.92	1.1	44.5	118.0	0.161	15.3
43	1.00	1.30	1.60	1.1	45.5	122.0	0.132	9.5
89	1.20	1.80	1.40	1.6	34.5	108.0	0.139	9.6
21	1.10	1.50	1.50	1.49	34.6	146.0	0.137	10.1
92	1.92	1.10	1.90	1.10	34.8	—	0.122	11.9
53	0.80	1.20	0.84	1.17	24.3	158.0	0.151	15.9
29	0.88	0.88	0.72	1.44	28.5	115.0	0.139	16.7
Av.....	1.11	1.28	1.30	1.24	35.9	141.0	0.147	13.1
B. Rats given sodium salicylate								
39	1.30	1.10	1.30	1.44	27.9	216.0	0.330	26.9
41	1.10	1.00	1.00	1.46	35.5	159.0	0.246	23.9
68	1.30	1.20	1.30	1.34	30.2	135.0	0.326	25.8
32	1.20	1.00	1.30	1.47	33.4	125.0	0.422	36.4
66	1.00	1.70	—	1.04	26.5	177.0	0.284	17.8
72	1.18	1.30	1.40	1.18	37.7	123.0	0.236	19.2
57	0.95	1.20	1.20	1.15	29.4	127.0	0.284	25.4
44	1.30	1.40	1.20	1.42	28.5	140.0	0.286	22.0
69	1.20	1.30	1.40	1.31	33.0	120.0	0.254	19.5
43	1.00	1.00	1.50	1.32	43.0	171.0	0.278	22.0
22	1.00	1.00	0.98	1.24	31.0	181.0	0.314	31.4
54	1.10	1.40	1.30	1.48	41.7	148.0	0.272	21.7
Av.....	1.14	1.22	1.26	1.32	33.2	152.0	0.294	24.3

¹ The blood uric acid samples were taken at the beginning, middle and end of the collection period.

U.F. equals urine flow in cc. per hour per 100 grams of body weight.

C.C. equals creatinine clearance in cc. per hour per 100 grams of body weight.

H.C. equals hippurate clearance in cc. per hour per 100 grams of body weight.

U.A. equals mgm. of uric acid excreted in urine per hour.

U.A.C. equals uric acid clearance in cc. per hour per rat.

The average blood allantoin (1.75 mgm. per 100 cc.) of the six rats which received salicylate remained approximately unchanged (1.77 mgm. per 100 cc.) during the clearance period. The average amount of allantoin excreted was 2.05 mgm. per

hour. On the basis of these results, it seems likely that the ingestion of salicylate had no significant effect either upon the blood or upon the renal excretion of allantoin.

3. *Acute effect of sodium salicylate and glycine upon the blood and urinary uric acid of the normal rat.* Uric acid clearances were done upon nine rats which not only received the usual amount of sodium salicylate but also 50 mgm. of glycine by mouth at the beginning of the collection period and five mgm. every hour of the clearance determination.

The combined administration of salicylate and glycine was followed by an increase in the amount of uric acid excreted without a preceding or concomitant abnormal increase in the blood uric acid. However, the average uric acid excretion of the nine rats was 0.318 mgm. per hour which was only about eight per cent higher than the excretory rate of rats receiving only salicylates (see table 1 B). Moreover the average uric acid clearance in this series was only 17.7 cc. per hour as compared to 24.3 cc. of rats receiving salicylate alone. Whether this lowered clearance represents an interference by glycine or whether it resulted because of the reduced urine flow is unknown. It would appear then that little if any augmentation was obtained in the excretion of uric acid by the combined administration of glycine and salicylate.

4. *Effect of sodium salicylate upon the blood uric acid and allantoin of nephrectomized rats.* Fourteen rats were nephrectomized after control determinations of blood uric acid and allantoin had been made. Five of these rats received 30 mgm. of sodium salicylate in three divided doses in the 24-hour period following nephrectomy. Blood samples were obtained again from each of the rats, 24 hours after nephrectomy, and analyzed for uric acid and allantoin.

The administration of sodium salicylate had no effect upon the blood uric acid of nephrectomized rats. The average blood uric acid of the nine control rats was 1.34 mgm. per 100 cc. before and 1.49 mgm. per 100 cc. 24 hours after nephrectomy. The blood uric acid of the five rats which received salicylate was 1.40 mgm. per 100 cc. before and 1.26 mgm. 24 hours after nephrectomy.

Salicylate moreover was found to have no effect upon the blood allantoin concentration of nephrectomized rats. Thus the average blood allantoin of the control rats was 2.10 mgm. per 100 cc. before and 37.37 mgm. per 100 cc. 24 hours after nephrectomy. The average blood allantoin concentration of the five rats which received salicylate was 35.6 mgm. per 100 cc., 24 hours after nephrectomy.

5. *Chronic effect of sodium salicylate upon the blood and urinary uric acid content of the normal rat.* Six rats were caged individually and the daily urinary output was recorded for seven days. Determinations also of the uric acid in the urine of these rats were made every other day during the same control period. The average daily uric acid output for a week's period was then calculated by multiplying the average daily volume of urine in cc. by the average uric acid concentration of the three urine samples analyzed. Likewise the blood uric acid of these rats was determined every other day and the average daily blood uric acid for a week's period was calculated by averaging the uric acid content of three blood samples analyzed during the control period.

After these control studies had been completed, each rat received 30 mgm. of sodium salicylate a day (in three divided doses) by stomach tube. Determinations of urinary and blood uric acid were obtained three times a week and averaged exactly as in the control period. The experiment was continued for 42 days after the control period.

Although the experiment began with six rats only three remained at the end of the sixth week, at which time the feeding of sodium salicylate was discontinued. The other three rats died following accidental perforation of their esophagus by the stomach tube during the first two weeks of the experiment.

As was expected (see table 2), the oral administration of salicylate was followed by a prompt increase in the amount of uric acid excreted per day. The average daily excretion of uric acid was 3.04 mgm. before and 4.43 mgm. (an increase of 45.7 per cent) during the first week of salicylate ingestion. As table 2 indicates the average daily uric acid excretion in subsequent weeks continued to be greater than that during the control period although never as large as that obtained

TABLE 2. CHRONIC EFFECT OF SODIUM SALICYLATE UPON THE URIC ACID CONTENT OF THE NORMAL RAT'S BLOOD AND URINE

DAYS	NUMBER OF RATS	BLOOD URIC ACID (MG/100 CC.)		URINE FLOW (CC/DAY)		URIC ACID EXCRETED (MG/DAY)	
		Average	Range	Average	Range	Average	Range
1-7 ¹	6	1.22	(1.1-1.5)	9.1	(6.5-11.0)	3.04	(2.5-3.74)
7-14	6	1.30	(1.2-1.4)	14.3	(12.7-17.0)	4.43	(3.66-4.90)
14-21	4	0.90	(0.8-1.0)	14.1	(11.6-20.2)	3.94	(3.34-4.20)
21-28	3	0.91	(0.8-1.1)	18.1	(15.4-20.7)	4.02	(3.82-4.29)
28-35	3	0.53	(0.5-0.6)	18.8	(17.6-21.2)	3.88	(3.77-4.09)
35-42	3	0.53	(0.5-0.6)	20.9	(17.0-26.1)	4.24	(4.02-4.60)
42-49	3	0.50	(0.5-0.5)	19.2	(12.6-26.9)	3.72	(3.17-4.07)

¹ Control period of seven days during which no salicylate was given.

during the first week of salicylate feeding. During the sixth week the average daily uric acid excretion of the remaining three rats was 3.72 mgm. (an increase of 22.4 per cent above the control value).

The average blood uric acid of the chronic rats was 1.22 mgm. per 100 cc. during the control period, decreased to 0.90 mgm. at the second week of salicylate administration and apparently became fixed at approximately 0.5 mgm. per 100 cc. from the fourth week on.

Coincident with the marked increase in uric acid excretion, the average daily urine volume increased from the control value of 9.1 cc. to 14.3 cc. during the first week of the experiment (see table 2). This increase in urine volume continued throughout the entire experiment.

DISCUSSION

Our experimental observations in common with those of previous investigators (1-5) clearly indicated that salicylate effected a marked and very rapid increase

in the excretion of uric acid. The inability of salicylate to cause a change in the blood uric acid preceding this increased urinary excretion either in the acute or chronic experiments suggested that the increase was not the result of a changed or accelerated purine metabolism. Furthermore, the inability of salicylate to effect any change in the renal excretion of allantoin (which also is a product of the rat's purine metabolism) added additional evidence to suggest that salicylate did not act upon the mechanisms primarily involved in purine metabolism. Finally, the inability of administered salicylate to produce any change in either the blood uric acid or allantoin concentration of nephrectomized rats left little doubt that the increased excretion of uric acid after salicylate was due solely to some sort of renal phenomenon.

The absence of any significant change in the creatinine and hippurate clearances or in the excretion of allantoin following the ingestion of salicylates indicated that the increase in urinary uric acid following the latter could not have been due to either an increase in renal blood flow or in rate of glomerular filtration. Thus it would seem most likely that salicylate, as Talbott (9) has postulated, acts to influence the renal tubular mechanism involved in the excretion of uric acid. In this regard, it acts similarly to glycine which also has been found (11) to interfere with the excretion of uric acid. It is quite possible that both of these substances act upon the same tubular mechanism. The inability of both substances when given together to increase the uric acid output significantly above that following the ingestion of salicylate alone might be due to the possibility that the latter drug, in the dosage used, effected a physiological maximum of tubular interference in this regard.

Whether this tubular mechanism is influenced by salicylate only when the renal innervation is intact, as stated by Gray and Grabfield (8), is not yet determined. Unfortunately, the control studies of these authors indicated an increase in not only the excretion of uric acid but also of allantoin and urinary nitrogen, following the ingestion of salicylate. These last two increases are very difficult to understand in view of the fact that the creatinine clearance of their animals remained unaffected by the administration of salicylates. Since the allantoin clearance has been found also to be a measure of glomerular filtration in both the rat and dog (15) it seems strange that this clearance should increase and the creatinine clearance (its exact counterpart) should remain unchanged. Likewise, the unexplained increase of urinary nitrogen (2-4 grams), after salicylate with no change in the creatinine clearance again, throws some doubt upon the accuracy of their control studies.

CONCLUSIONS

1. Sodium salicylate was found to effect an immediate increase in the urinary excretion of uric acid of the normal rat without a preceding or concomitant change in the blood uric acid, the renal plasma flow or the glomerular filtration rate.
2. Sodium salicylate was found to exert no effect either upon the blood or urinary allantoin content of the normal rat.

3. Glycine was not found to augment significantly the effect of sodium salicylate in increasing the excretion of uric acid.

4. Sodium salicylate was unable to cause any significant change in the blood uric acid or allantoin of the nephrectomized rat.

5. The chronic administration of sodium salicylate was found to cause an immediate and continued increase in uric acid excretion. The blood uric acid of such treated animals progressively fell to approximately one third of its control value.

6. It was concluded that salicylate acts to increase the output of uric acid by interfering with the tubular mechanism concerned with the excretion of uric acid.

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EFFECT OF FOLIC ACID AND LIVER EXTRACT ON SERUM AND RED CELL CHOLINESTERASE ACTIVITY

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Received for publication December 15, 1947

The irreversible inactivation of cholinesterase (ChE) by di-isopropylfluorophosphate (DFP) has made possible numerous studies of the rates of regeneration of red cell and plasma ChE. It has been shown, in animals treated with DFP, that red cell ChE is restored at a rate commensurate with erythropoiesis and that plasma ChE restoration proceeds at a rate closely similar to that of the regeneration of other plasma proteins (1, 2). It is probable that the regeneration rate of a given tissue ChE after DFP reflects the protein production rate characteristic of that tissue.

Recent reports (3-5) state that liver extract and folic acid are effective in the treatment of anemia by virtue of an alleged capacity for enhancing serum ChE activity both *in vitro* and *in vivo*. This increase in serum ChE activity is said to occur both in normal animals and in those with reduced ChE levels. The experiments reported herein were prompted by interest in the alleged role of ChE in erythropoiesis, the fundamental interest which attaches to these observations with respect to protein synthesis in general, the mechanism of action of drugs—in either activating a specific protein, viz., ChE or in accelerating its restoration in the body—and the possible therapeutic value of folic acid or liver extract in poisoning by anti-cholinesterase agents.

METHODS

1. *In vitro experiments.* Blood plasma samples were obtained from normal humans, normal dogs, and dogs chronically poisoned with DFP. To these were added varying concentrations of folic acid¹ or liver extract and incubation was carried out for 2½ hours at 37°C. in a constant temperature bath. Plasma ChE activity was determined manometrically as previously described in application to a study of human liver disease (6).

2. *In vivo experiments.* Four normal female dogs were used; they were maintained in good health on a diet constant in composition prior to and during the study. Determinations were made of plasma ChE activity, erythrocyte counts, hemoglobin percentages and reticulocyte percentages. The Haden-Hausser hemoglobinometer was used for determining hemoglobin percentages; reticulocyte counts were made on blood smears stained with brilliant cresyl blue and counterstained with Wright's stain.

The solutions of folic acid, acetylcholine bromide and physostigmine were made in distilled water; the acetylcholine solution was buffered with sodium phosphate

¹ The folic acid used was supplied by Lederle Laboratories.

to reduce irritation at the site of injection. Armour's 'Liver Liquid Parenteral' was used; it contains 15 U.S.P. units in 1 cc.

RESULTS

1. *In vitro*. The incubation of normal human plasma with relatively large concentrations of folic acid or liver extract produced no significant change in ChE activity from control figures. An example of such results appears in table 1.

TABLE 1. EFFECT OF FOLIC ACID AND LIVER EXTRACT ON CHE ACTIVITY OF NORMAL HUMAN PLASMA

INCUBATION MIXTURE	ChE ACTIVITY (MM ³ CO ₂ /0.1 cc/30 MIN.)
1.0 cc. serum.....	217
1.0 cc. serum +0.2 mgm. FA.....	219
1.0 cc. serum +0.08 u. LE.....	209
1.0 cc. serum +0.75 u. LE.....	217

TABLE 2. EFFECT OF FOLIC ACID AND LIVER EXTRACT ON THE PLASMA CHE ACTIVITY OF DOGS TREATED WITH DFP

DOG	INCUBATION MIXTURE	ChE ACTIVITY (MM ³ CO ₂ /0.1 cc/30 MIN.)
1	1.0 cc. plasma	30
	1.0 cc. plasma + 0.2 mgm. FA	28
	1.0 cc. plasma + 0.5 mgm. FA	33
	1.0 cc. plasma + 1.0 mgm. FA	31
2	1.0 cc. plasma	67
	1.0 cc. plasma + 10.0 mgm. FA	68
	1.0 cc. plasma + 2.5 u LE	78
2	1.0 cc. plasma	68
	1.0 cc. plasma + 10 mgm. FA	67
	1.0 cc. plasma + 2.5 u LE	67
2	1.0 cc. plasma	79
	1.0 cc. plasma + 0.75 u LE	79
	1.0 cc. plasma + 0.16 u LE	78
3	1.0 cc. plasma	37
	1.0 cc. plasma + 0.2 mgm. FA	39
	1.0 cc. plasma + 0.75 u LE	35

Plasma samples from 3 dogs chronically poisoned with DFP, possessing 40 per cent or less of their normal (pre-DFP) ChE activity, were similarly incubated with folic acid and liver extract in wide ranges of concentration. In no instance did these drugs produce a significant change in the ChE activity. These results are shown in table 2.

2. *In vivo*. In an attempt to produce a macrocytic anemia and a lowering of plasma ChE activity (4), two dogs were given daily subcutaneous injections of 3.0 mgm. of acetylcholine bromide and 1.0 mgm. of physostigmine salicylate for a

period of 61 days; for the same period two dogs were kept as untreated controls. In neither of the two treated dogs were there any significant changes in plasma ChE activity, erythrocyte counts, reticuloocyte percentages and hemoglobin percentages. In addition, there was no appreciable difference between the data obtained on these dogs and those obtained on the control animals.

During the subsequent 25-day period the acetylcholine and physostigmine injections were continued; in addition, although no anemia had been produced, one of the experimental animals and one of the control animals received a daily intra-

TABLE 3. EFFECT OF DRUGS ON BLOOD CONSTITUENTS

DOG	EXPERI- MENTAL PERIOD	DRUGS	RED CELLS			HEMO- GLOBIN	RETICU- LOCYTES	ESTERASE ACTIVITY (MM ³ CO ₂ /0.1 cc/30 MIN.)	
			Detn.	Millions				Av.	Extremes
				Av.	Extremes				
1	days					grams %	per cent		
	13	none	4	5.37	5.15-5.81	12.5	0.1	128	125-130
	61	AcCh, Phys	13	5.82	5.14-6.81	12	0.1	118	101-141
	25	AcCh, Phys	3	6.05	5.58-6.49	13	0.2	108	84-116
		LE							
	12	AcCh, Phys FA	2	5.82	5.52-6.12	14.5	0.1	109	105-113
2	13	none	4	5.57	5.14-6.01	13	0.1	77	72- 78
	61	AcCh, Phys	13	6.09	5.16-6.44	13	0.2	75	55- 91
	25	AcCh, Phys	3	5.75	5.17-6.19	14	0.1	71	66- 75
	12	AcCh, Phys	2	6.06		14	0.2	71	70- 72
		FA							
3	13	none	4	6.69	6.10-7.38	15	0.2	81	78- 82
	61	none	13	6.83	5.82-7.48	14	0.2	74	59- 90
	25	none	3	5.89	4.95-6.70	14	0.2	78	73- 82
	12	none	2	6.50	5.99-7.00	14	0.1	74	72- 75
4	13	none	4	6.56	5.84-7.33	15	0.2	73	71- 74
	61	none	13	6.84	5.89-7.30	14.5	0.2	74	61- 89
	25	LE	3	6.74	6.62-6.89	14.5	0.1	81	74- 84
	12	none	2	6.57	6.32-6.82	14.5	0.1	78	76- 80

muscular injection of two units of liver extract. Throughout this 25-day period no significant changes were noted.

In order to test the possible effect of folic acid on the blood constituents the experimental dogs were placed on a final 12-day period during which they received daily an intramuscular injection of two mgm. of folic acid in addition to their usual acetylcholine and physostigmine. This procedure also proved fruitless; no significant changes were noted in any of the collected data.

The combined data on the four dogs for these experimental periods are shown in table 3. Although there was considerable variation in the red cell counts from

day to day, no trend toward anemia was discernible. The esterase data likewise showed a variability over the three-month period but no trends were established.

A second series of experiments was carried out to test the validity of the thesis that folic acid and liver extract hastened the generation of plasma ChE. In this series the regeneration of plasma ChE was followed after the enzyme had been reduced to low levels by doses of DFP. Of the four dogs given DFP, two were allowed to continue without further treatment, one was given a daily injection of two mgm. of folic acid, and one was given a daily injection of two units of liver extract. The contour and time course of the recovery curves of plasma ChE in the dogs receiving liver extract and folic acid did not differ from those of the control dogs. The curves are shown in figure 1.

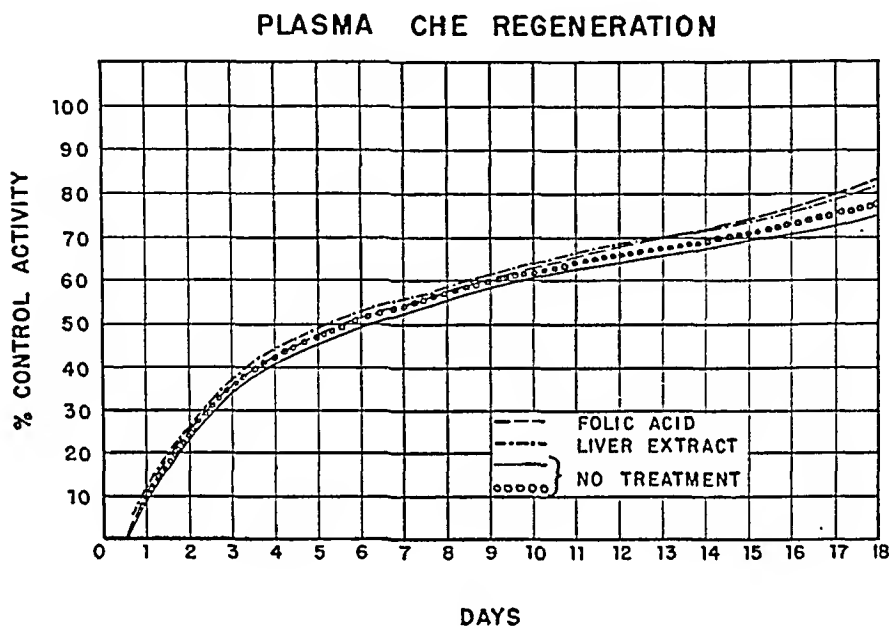


FIG. 1

DISCUSSION

Although the experiments are limited in number, these results do not support the idea that the ChE activity of both normal and ChE-depleted plasma may be affected *in vitro* by either folic acid or liver extract. Indeed, it seemed unlikely that a protein enzyme could be generated from precursors by incubation, but the possibility remained that folic acid or liver extract might act as ChE activators. This possibility has been discounted in the present experiments.

That the plasma ChE activity of normal dogs and of dogs with plasma ChE severely depleted by DFP may be influenced by parenteral administration of liver extract and folic acid is likewise not supported by the present experiments. Further study may be necessary to resolve the discrepancy between these and other published observations (3-5), but it is doubtful that liver extract or folic acid will prove valuable therapeutically for augmenting ChE activity in cases of

poisoning with anti-ChE compounds. The reason for the discrepancy between our results and others is not obvious, for differences in methods of ChE determination are not recognized as giving such widely divergent results, at least in serum and erythrocytes. The ChE activities which we encountered were such that they lay in a range of our method sufficiently sensitive to make impossible a failure to detect increases of the order claimed by previous workers as due to liver extract or folic acid. Further, this circumstance also prevented small fortuitous changes from appearing as large percentage changes. How these matters stand with the titrimetric procedure used by others in this particular problem is difficult to assess from the published data. Hence, we can offer no explanation for the discrepancy.

Considerable variation was noted in the erythrocyte counts of the control and experimental animals, but no trend toward anemia was discernible as a result of acetylcholine and physostigmine administration in doses reportedly effective in this respect. Although choline feeding (4) was not employed in these experiments, the consistently negative character of the results in the problem as a whole does not lead to the expectation that choline might produce anemia or reduction in ChE activity. In this respect, other investigators (7) have been unable to produce a macrocytic anemia in dogs fed choline or choline and fat.

These considerations do not support the hypothesis that the acetylcholine-ChE mechanism is concerned with erythropoiesis. Recently published results of other investigators (2) in patients with pernicious anemia have proven that ChE is not concerned with erythropoiesis. Indeed it is much more logical to expect that any increase in the plasma ChE activity in patients treated for pernicious anemia is a resultant of recovery and not the cause of it.

CONCLUSIONS

1. Liver extract and folic acid *in vitro* and *in vivo* do not increase plasma ChE activity in normal or plasma ChE-depleted dogs.

2. Parenterally administered acetylcholine and physostigmine do not induce erythrocyte or hemoglobin changes suggesting anemia in dogs.

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BLOOD PRESSURE CHANGES IN RESPONSE TO ELECTRICAL AND CHEMICAL (ACETYL-BETA-METHYLCHOLINE) STIMULATION OF THE CEREBRAL CORTEX IN DOGS

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Received for publication March 7, 1947

The cortical representation of autonomic functions has been the subject of a number of recent investigations, which may be roughly classified under the following categories: *a*) studies of the autonomic effects of electrical cortical stimulation; *b*) studies of the autonomic effects of well-defined ablations; *c*) the recording of action potentials from the brain surface during stimulation of the vagal nerve; *d*) neurohistological studies of the intracerebral pathways to establish the relations of the allocortex with the hypothalamic centres.

Most experiments can be classified under the first heading. Although they all yield evidence of a cortical component in the regulation of autonomic functions, the use of different experimental animals often makes it difficult to compare the results as regards the exact location of such cortical action. Also, the information gained in this way is far from being unequivocal. Thus, Hoff and Green (1), investigating the anterior and posterior sigmoid gyri in cats under ether anaesthesia, observed an elevation of blood pressure in response to electrical stimulation of this area, together with a dilatation of the pupils. This was confirmed in later experiments (2), in which, moreover, a simultaneous diminution in kidney volume and an increase in limb volume could be recorded, suggesting that a shift of blood from the visceral to the muscular bed is the underlying mechanism for the cortically induced blood pressure rise. Similar blood pressure responses were obtained by Crouch and Thompson (3) from the anterior sigmoid in cats (Dial, nembutal, ether anaesthesia). In dogs, however, they observed mainly a fall in blood pressure, while Spiegel and Hunsicker (4) obtained the blood pressure fall also in cats (ether anaesthesia). An earlier publication by Dusser de Barenne and Kleinknecht (5) described a fall in blood pressure upon stimulation of the motor cortex in cats, dogs and rabbits, while in 1942 Siao-Hsi *et al.* (6) again could obtain only depressor effects following stimulation of this area in dogs under ether-chloralose anaesthesia. Also, in further contrast to Hoff and Green's observations, they found the kidney volume increased. Bucy and Case (7), in a study of the cortical innervation of respiratory movements, mention rather inconstant blood pressure changes. One may conclude, while accepting the evidence of a vasomotor function of the sigmoid gyri, that we have no reliable information as to its exact character.

In this study concerned with the vasomotor effects of cortical stimulation, mention should be made also of blood pressure effects obtained from areas other than the sigmoid gyri. Hoff and Green (1) reported the presence of depressor points, diffusely scattered over the convexity of the hemispheres in cats, and

of a pressor area in the superior precentral cortex in the macaque. Smith (8), who located an inhibitory effect on respiration in the lateral part of area 6 (cats, dogs) or 6b (monkey), stated that such inhibition was usually accompanied by a marked rise in blood pressure (ether anaesthesia). Bailey and Sweet (9) found the blood pressure noticeably raised upon stimulation of area 13 on the orbital brain surface of the macaque and of a similar region in the cat's brain (nembutal anaesthesia). This area had been previously described by Bailey and Bremer (10) as the sensory cortical end station of vagal nerve impulses.

The discrepancies in vasomotor responses obtained by the different authors upon stimulation of identical areas on the brain surface may find their explanation in the variability of a number of factors. Of these, some are unrelated to the animal itself, such as the kind of anaesthesia used, the depth of anaesthesia, the type of stimulation applied, and the strength of stimulation. Other factors are directly related to the animal, such as species and age, the general physiological state of the animal, especially with regard to the activity of the gastro-intestinal tract, the initial blood pressure at the start of the experiment, and the excitatory state of the brain surface which may change considerably during the course of the experiment.

In this study, the usefulness of a different type of stimulus, the local application of acetyl-beta-methylcholine, has been investigated and compared with that of electrical stimulation.

METHOD

All dogs, of different race, age and sex, received their last feeding in the evening previous to the day of operation and experimentation. Anaesthesia was induced by intraperitoneal injection of Dial, 45 to 50 mgm. per kgm., usually administered in two or three separate doses before the operation in order to avoid too deep an anaesthesia. In two dogs Na-amytal, 50 mgm. per kgm., was used.

After ligation of both common carotids, the frontal, parietal and most of the occipital and temporal lobes were exposed on either the left or right side, while in the majority of cases, one eye was enucleated in order to provide a better approach to the orbital brain surface. Before the dura was opened, one common carotid was connected with a mercury manometer, a 7 per cent Na-citrate solution being used as mediator between blood and mercury column. From the moment the dura was opened, the brain surface was kept wet by application of warm Ringer's solution.

In coagulating the connections of pial vessels with the sagittal sinus, the use of a monopolar 'Highfrecator' needle proved to be of great advantage; for such purposes this simple instrument must be considered far superior to the generally used electrocoagulator of lower frequency, as supplied with electrosurgical units.

In seven dogs the cortex was stimulated faradically with bipolar copper wire electrodes 1 mm. apart, while the current was supplied by a 3-volt battery, connected with a Harvard inductorium, using a coil distance varying between 5 and 8 cm. Each stimulation lasted from 10 to 15 seconds. In four dogs the cortex was stimulated with a 2.5 per cent solution of acetyl-beta-methylcholine, applied

by means of minute pieces of filter paper soaked with this solution. The duration of the application varied from 40 seconds to 2 minutes.

RESULTS

In six of the animals, anaesthetized with Dial, blood pressure effects were easily obtained from either the anterior or posterior sigmoid gyrus or from both. A 7-8 cm. coil-distance of the Harvard inductorium provided an appropriate stimulus to provoke the effect from a fairly limited area. Stronger stimuli often elicited vasomotor responses from a far larger region, extending laterally into the coronal and ectosylvian gyri. The latter responses were usually accompanied by strong movements of the limbs and trunk, sometimes resulting in vigorous movements of the entire body. Therefore it was decided to consider only the effects of the weaker stimuli, with a coil distance of 8 cm. These, as well as the responses to stronger stimuli, always demonstrated themselves as a fall in blood pressure (fig. 2). The following characteristics were observed. Usually the depressor effect amounted to 15 to 20 mm. Hg, while in case of a high initial blood pressure a greater drop might occasionally be observed, not exceeding 30 to 35 mm. Hg. The drop started abruptly after a latent period varying between 6 and 9 seconds. It reached its lowest point within six seconds, but would never remain there for more than 2 to 3 seconds. From there it returned to its former level, in such a way that the more this was approached, the slower the rise would be. The whole phenomenon lasted from 20 to 25 seconds and was not influenced noticeably by the duration of the stimulus. Often upon stimulation of the sigmoid gyri, very slight movements of one of the opposite limbs were observed, while stimulation of the frontal part of the gyrus compositus anterior elicited rhythmic movements of the jaw with great regularity but with a considerable latency. Also from the latter area, the respiration was inhibited, while from the motor area the respiratory movements were intensified and accelerated. The results of the experiments in *dogs I, V and VI* are recorded in drawings of the stimulated areas in figure 1.

In *dog VII* a different anaesthesia was used, i.e., Na-amytal 50 mgm. per kgm. In this animal the respiratory effects were as easily obtainable as in the previous dogs. For vasomotor responses, however, stronger stimuli had to be used (coil-distance 5 to 6 cm.), and responses were evoked from a much wider area. Also, although never exceeding a rise of 5 mm., an elevation of blood pressure was observed equally as often as a drop, the latter usually being more pronounced than the former. Altogether it showed the picture of a lowered excitability for vasomotor responses which, when obtained, were inconstant and not sharply limited to one area, as compared with dogs under Dial anaesthesia. Figure 1 illustrates the findings in this animal (*dog VII*), although the drawing should not be compared with those for the other animals, since the stimulation was stronger and the responses weaker.

In an attempt to find a more physiological stimulus than those hitherto used, several chemical compounds were applied to the brain surface during the course of two of these experiments. The following were used: adrenaline, ephedrine, strychnine, prostigmin, and acetyl-beta-methylcholine. Only acetyl-beta-

methylcholine evoked a marked vasomotor response, and it was considered to be of importance to investigate the possibilities and characteristics of this substance as a cortical stimulant in the study of blood pressure regulation.

In three animals anaesthetized with Dial, and in a fourth one anaesthetized with Na-amytal, it was demonstrated that this drug, if applied in a concentration of ten per cent, produced a marked depressor effect from a wide area. Concentrations of 2.5 per cent would still yield a marked response, but the action became very closely limited to a small surface region. Concentrations lower than two per cent usually remained without effect in the investigated areas. Therefore

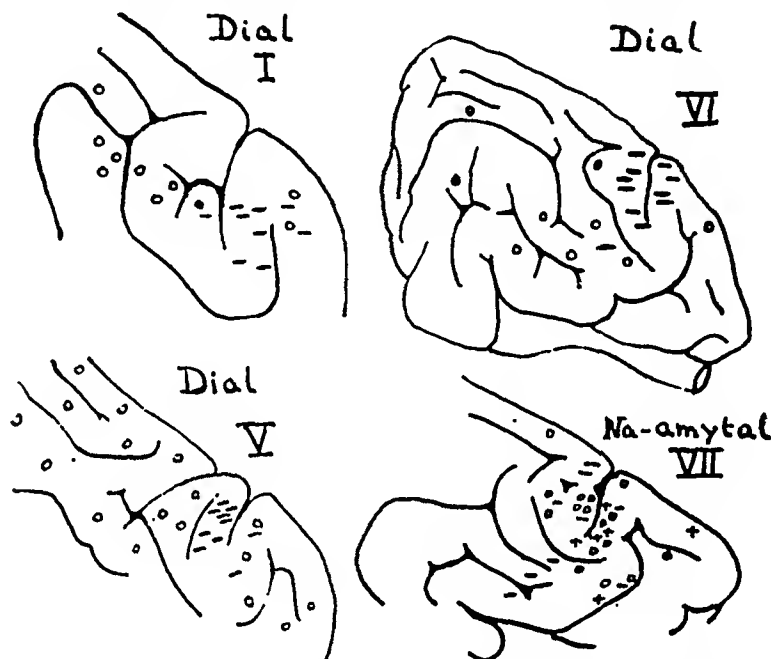


FIG. 1. BLOOD PRESSURE EFFECTS in response to faradic cortical stimulation. The drawings show the stimulated area around the cruciate sulcus in dogs I, V and VI (Dial-anaesthesia) and in dog VII (Na-amytal anaesthesia).

0 means: no blood pressure response; + means: elevation of blood pressure; - means: fall of blood pressure.

in the following experiments a concentration of 2.5 per cent has been used without exception.

Two responsive areas were located: one in the posterior sigmoid gyrus near the midline (figs. 3 C, 4 C, 5 I, 7 C), the other in the anterior ectosylvian gyrus (figs. 6, 7 M). There was always a conspicuous drop in arterial pressure. The following characteristics could be observed: the depressor effect varied between 30 and 50 mm. Hg; the drop started abruptly, sometimes immediately preceded by an inconspicuous rise (fig. 6) after a latent period, varying between 25 to 40 seconds. During this drop, pronounced vasomotor waves of the third order¹

¹ The term 'third order waves' designates wave-like changes in the carotid blood pressure curve as compared to the 'first order' and 'second order' waves, respectively, representing the heart beat and the respiration. The first order waves are superimposed on the second order waves; the first and second order waves are superimposed on the third order waves.

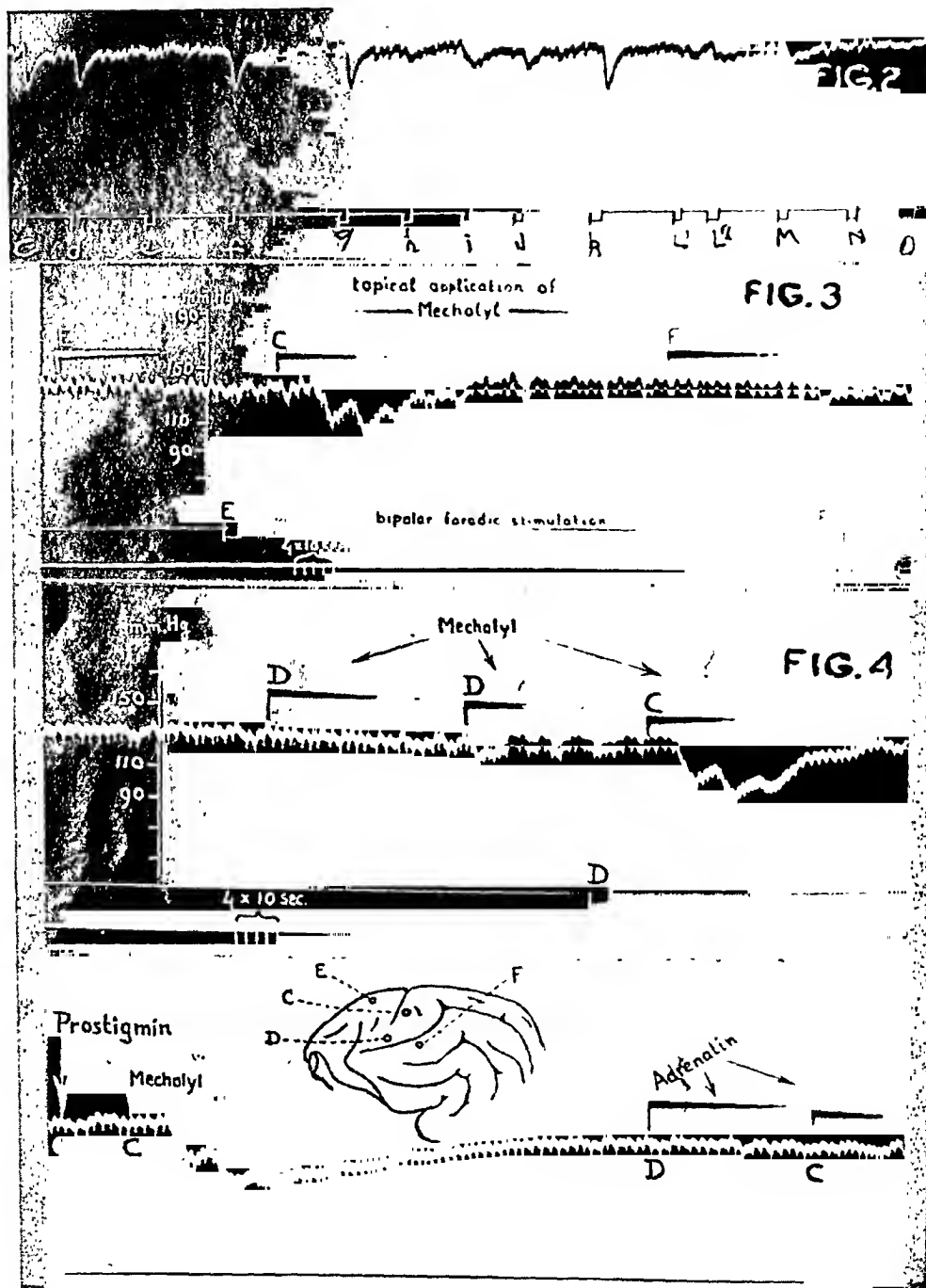


FIG. 2. CHARACTERISTIC BLOOD PRESSURE RESPONSES to bipolar faradic stimulation in a Dial-anaesthetized dog (*dog III*).

FIG. 3. CHARACTERISTIC EFFECT with third order waves obtained by acetyl-beta-methylcholine application to responsive area C. Similar or electrical stimulation of areas E and F remains without effect (*dog A* same as in fig. 4).

FIG. 4. BLOOD PRESSURE CHANGES in response to local applications of acetyl-beta-methylcholine, prostigmin, prostigmin + acetyl-beta-methylcholine, adrenalin. The two blood pressure tracings are continuous. The capitals above the tracings correspond with the homonymous spots on the drawing of the cerebral cortex. Note that only C is responsive to acetyl-beta-methylcholine and that this response is considerably prolonged by simultaneous application of prostigmin, while adrenalin fails to elicit any blood pressure changes. The appearance of third order waves is clearly visible (*dog A*).

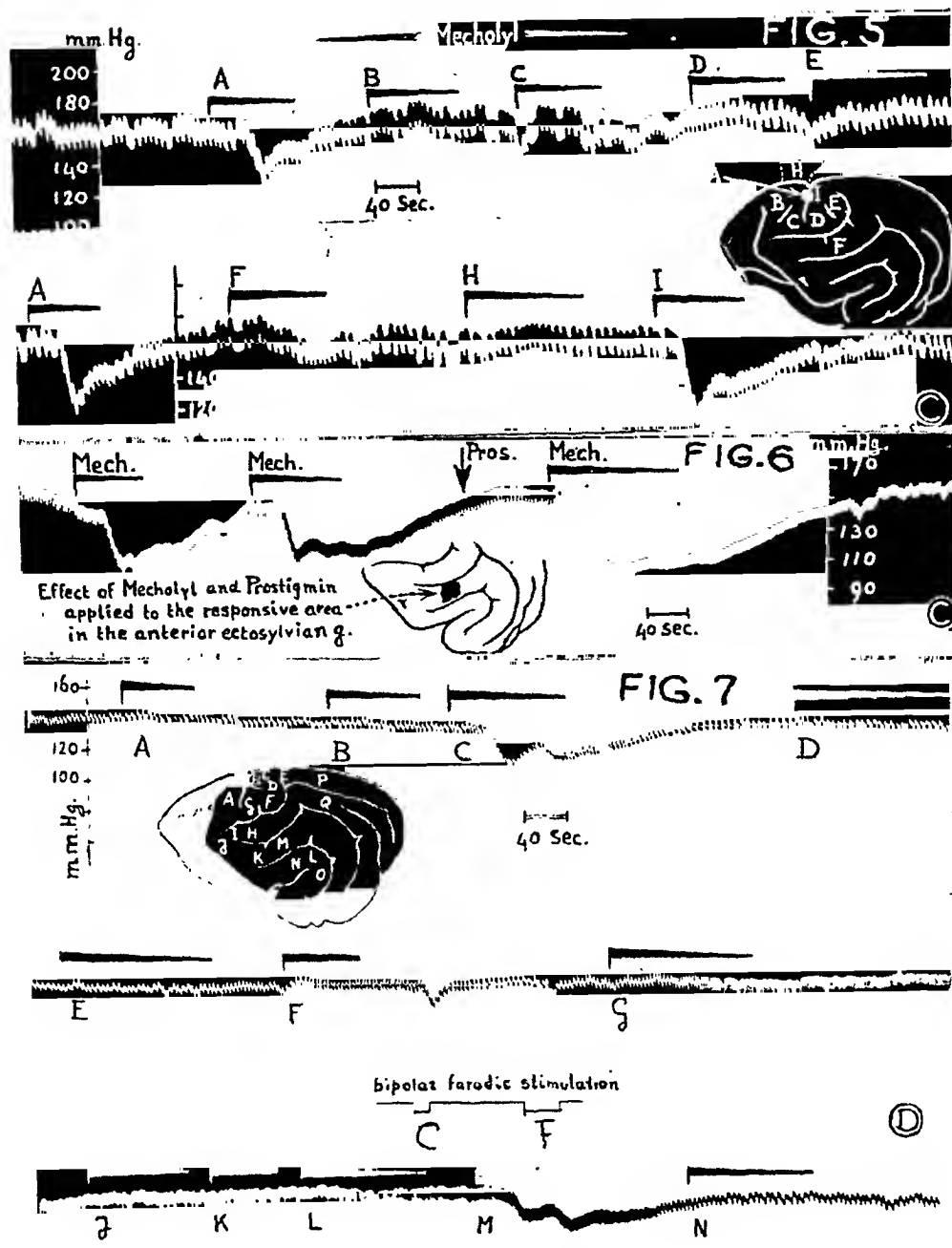


FIG. 5. UPPER AND LOWER TRACINGS ARE CONTINUOUS. The response from area A is probably due to stimulation of that part of the cortex which borders the cruciate sulcus posteriorly. A stronger effect was obtained from area I (dog C).

FIG. 6. STIMULATION OF ANTERIOR ECTOSYLVIAN GYRUS with acetyl-beta-methylcholine and prostigmin (dog C).

FIG. 7. THE TWO UPPER TRACINGS WERE CONTINUOUS. Responsive areas are found in C and M. Compare the effect of acetyl-beta-methylcholine stimulation with that of electrical stimulation of area C in the upper and middle tracing (dog D).

appeared with great constancy, at a rate of about one per 40 seconds. The lowest pressure level was reached in either the first or the second wave. Usually at

least three waves could be recorded. The blood pressure then slowly returned to its former level. The whole phenomenon lasted from 2 to 3 minutes. A second application, rapidly succeeding the first one, produced a somewhat longer lasting effect, which may have been due to local cumulative action or to facilitation (fig. 6).

In all experiments the response was markedly restricted to a well circumscribed area. Immediately adjacent spots remained completely silent, even when repeatedly stimulated. Moreover, the responses were much more pronounced than those obtained by electrical stimulation of the same region.

In order to provide further proof for the cortical action of the drug, prostigmin salicylate powder was applied to the responsive spots. Although this substance did not evoke any vasomotor response, it was able to enhance and prolong conspicuously the action of consecutively applied acetyl-beta-methylcholine. On the other hand, locally applied pontocaine-HCl crystals could forestall and abolish the acetyl-beta-methylcholine effect.

It was further observed that, due to factors somehow related to the exposure, the brain surface showed considerable changes in its physiological state within one to one and one half hours after the dura had been opened, notwithstanding the fact that the cortex was kept wet continuously with Ringer's solution. Either all responses would disappear, or, as happened in one case, responses were obtained from almost any point on the exposed surface, previously silent, while freshly exposed parts still behaved normally.

One animal was anaesthetized with Na-amytal (Dog D). Here acetyl-beta-methylcholine showed the same effect, with the restriction that the blood pressure fell to a lesser degree, although the duration of the phenomenon remained the same, as compared with the other experiments.

DISCUSSION

When the vasomotor action of bipolar faradic stimulation was compared with that of local application of acetyl-beta-methylcholine the following facts were noted:

	FARADIC	ACETYL-BETA-METHYLCHOLINE
latent period	6 to 9 seconds	25 to 40 seconds
effect	drop in arterial pressure	drop in arterial pressure appearance of third order waves
character of drop	abrupt	abrupt
difference between highest and lowest level	15 to 20 mm. Hg	30 to 50 mm. Hg
site of action	fairly well restricted to circumscribed area	restricted to more sharply defined and smaller area
duration of the phenomenon	20 to 25 seconds	2 to 3 minutes

The difference in duration between the effects of both types of stimulation is of special interest, since continuation of the faradic stimulus did not appreciably prolong the effect. For that reason, the much longer-lasting response to acetyl-beta-methylcholine application cannot be attributed merely to a difference in duration of the stimulus; and although it is quite impossible to determine exactly the termination of the latter, it seems very likely that it involves a different mechanism than the former. This is further suggested by the appearance of vasomotor waves of the third order.

In this respect mention should be made of the work of Brenner and Merritt (11) and Forster and McCarter (12, 12a), who both draw attention to the similarity between the action of choline derivatives on the electrical activity of the cortex and the characteristic phenomena of cortical activity in epileptic patients. In 1937 Fiamberti (13) had produced generalized convulsions in man by means of intracisternal injections of acetylcholine. Williams (14) observed increased abnormal activity of the electroencephalogram upon intravenous injection of acetylcholine in epileptic patients.

Miller, Stavraký and Woonton (15) found no changes in the rabbits' electroencephalogram after injection of one per cent acetylcholine unless the animals were physostigminized. Brenner and Merritt (11) showed that this concentration was too low to be effective but that definite responses could be obtained with concentrations above 2.5 per cent. In cats, they also determined the liminal concentrations for acetyl-beta-methylcholine (1.5 per cent) and for carbamylcholine (0.3 per cent).

Local applications of acetylcholine to the cat's cortex (Chatfield and Dempsey, 16) were ineffective in a concentration of one per cent as shown in electrocorticographic studies of the somesthetic areas. In similar studies of the acoustic cortex in cats, Forster and McCarter (12) found acetylcholine highly effective in concentrations of 5 to 10 per cent. They made an interesting comparison with the cortical phenomena in epilepsy and their observation of self-continuing, intermittent, rapid electrical discharges ('Ach. discharges') may possibly explain the long-lasting effect of acetyl-beta-methylcholine in the experiments presented here.

From these data it would appear that acetylcholine, whether applied locally or administered systemically, increases the normally existing activity of the cortex, and one may apply this conclusion also to the action of acetyl-beta-methylcholine, since this drug differs pharmacologically from acetylcholine only in the respect that it is more slowly inactivated by cholinesterase.

Does this mean that the constantly obtained depressor effect reflects the true function of the cortical areas studied? It is thought that it does, but with the restriction that this function is conditioned by the actual status of the entire organism. In this respect one may regard as deciding factors *a*) the initial blood pressure, which in these experiments was above normal, probably because of the ligation of both common carotids; and *b*) activity or resting state of the gastrointestinal tract. In these experiments, the animals had received no food for at least 12 hours; thus the gastro-intestinal tract was left fairly inactive, and favourable conditions were provided for a shift of blood from the muscular to the visceral bed.

It is, however, quite conceivable that under different conditions, as for instance a low initial blood pressure, the true regulatory function of the cortical centres would have demonstrated itself in a pressor response instead of the drop observed here.

Finally, a selective influence of the anaesthetic upon one phase of the cortical regulatory action must be considered, but should not be overemphasized as an explanation for experimental controversies. Up to the present, data concerning such influence have been rather contradictory, often obtained without due consideration of the many other varying factors which have been discussed above. In our experiments, the slight vasopressor effects observed in one animal anaesthetized with Na-amytal may possibly be regarded as the initial rise of an abortive drop, while their absence in Dial anaesthetized preparations certainly does not mean that the anaesthesia had abolished pressor responses, as these could still be obtained without difficulty from the hypothalamic centers.

SUMMARY

In experiments on 11 dogs, the usefulness of acetyl-beta-methylcholine as a cortical stimulant in the study of cortical blood pressure centers has been investigated and discussed.

Acetyl-beta-methylcholine proved to evoke a clear response from two circumscribed cortical areas: one in the motor cortex, the other in the anterior ectosylvian gyrus.

The response demonstrates itself by a drop in arterial pressure of 30-50 mm. Hg, with a latency of 30 to 40 seconds and a duration of 2 to 3 minutes, further characterized by the appearance of vasomotor waves of the third order.

Simultaneous application of prostigmin prolonged the effect considerably. The use of Na-amytal as a general anaesthetic instead of Dial affected only the stimulability of the cortex, but did not change the character of the blood pressure response.

Acetyl-beta-methylcholine may be considered to be a useful cortical stimulant, since it probably approaches the ideal physiological stimulus more than does electrical faradic stimulation.

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SPECIFIC SYMPATHOMIMETIC SUBSTANCE IN THE BRAIN¹

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Received for publication September 23, 1947

Knowledge of biologically active chemical constituents of the central nervous system, possessing a possible significance for cerebral function itself and for the principles of psychosomatics and psychochemistry, is very scant and fragmentary. Vasodepressant substances have been extracted from the brain by several investigators (1-6). Their chemical nature has not been identified, but it is certain that at least one of them is not identical with either histamine, choline or adenylic acid. Von Euler (6) believes that the 'substance P', described by him, is a protein. All authors found the highest concentrations of vasodepressant substances to be present in the basal ganglia (1, 2, 3, 5).

Other biologically active substances which appear to be closely related to, or even identical with, posterior pituitary hormonal fractions have been demonstrated in the tissue of the hypothalamus: the oxytocic principle (7, 8) the anti-diuretic principle (8, 9) the blood-fat depressant principle ('lipoitrin,' 10) and the pigment hormone (11, 17).

The only statement regarding sympathomimetic vasopressor material in the brain has been made by von Euler (30), who found a very small amount of it in the brain of a calf and believes that it originates in vasomotor nerves, accompanying the brain vessels.

Numerous publications are concerned with the presence in the cerebrospinal fluid of biologically active material which is being attributed, in most instances, to secretory discharges from the posterior pituitary lobe. The following effects were found to be elicited either by the native cerebrospinal fluid or by extracts made from it: contractions of the uterus (12-15), stimulation of the intestines (16), inhibition of diuresis (15), expansion of melanophores (13, 17, 51) and elevation of the blood pressure (15, 16, 18, 19, 20, 26, 40). Stimulation of the hypothalamus is believed to be followed by an accumulation of pitressin or similar substance in the ventricular fluid (21). Most investigators agree that the highest concentrations of the respective active principles are present in the ventricular and cisternal fluid, while the lumbar fluid contains much smaller amounts, if any.

Page (20) described a vasopressor substance which he obtained through alcoholic extraction from ventricular and cisternal fluid of animals and man. He designated it as 'C.E.A.' (central excitatory agent).

¹ This study was aided, in part, by a grant from the John and Mary R. Markle Foundation.

Using a slight modification (22) of Shaw's (23) colorimetric method for the determination of epinephrine and related compounds, the writer has found a substance with epinephrine-like chromogenic and adsorbability properties in the brains of rats (24)² and later in the brains of other mammals and humans, and in the human cerebrospinal fluid.

In the following, the results of quantitative and qualitative colorimetric and pharmacological studies of this substance are reported. The term 'encephalin' will be used to distinguish it from epinephrine and sympathin.

METHODS

Colorimetric determination of encephalin. Fresh brains were carefully freed of meninges and grossly discernible blood vessels, including the choroid plexus. Sections from various parts of the brain, weighing around 800 mgm., sections of the cerebellum, cord, choroid plexus and whole pituitary glands were ground with Ottawa sand in 10 per cent trichloroacetic acid, filtered, and the filtrates worked up as described elsewhere (22). For average values of the entire brain, the cerebellum and the posterior half of the pons were removed, the hemispheres were ground in a Waring blender and about 800 mgm. of the resulting mash were used for analysis.

Lumbar fluids from live persons and cisternal fluid from cadavers (4 cc. each) were worked up in the same way as the brain filtrates (22). Also protein-free brain dialyzates, redissolved dry evaporation residues, adsorbates and eluates of various types (see below) were analyzed in the same fashion, going through both adsorptions by $\text{Al}(\text{OH})_3$ at pH 4.0 and 8.5. The acid $\text{Al}(\text{OH})_3$ adsorption process was omitted, however, when aqueous solutions of epinephrine or arterenol (nor-epinephrine) were analyzed for comparison.

The colorimetric encephalin readings are expressed in gamma equivalents, each gamma equivalent corresponding to the color effect of one gamma of epinephrine.

Isolation and purification of encephalin from brain tissue. This was done essentially by dialysis adsorption and elution. At first the dialyses were carried out according to the method described by O. Loewi (27) and by Cannon and Lissák (28). Brain tissue was ground with sand; distilled water (3 cc. per gram of brain) was added; dialysis followed for four hours in a shaking apparatus in special dialysis cups through a cellophane membrane against equal amounts of distilled water. The dialyzate was then evaporated in a desiccator at room temperature. The dry residue was redissolved in distilled water (5 cc. for 10 gram of fresh brain). With this procedure 46-51 per cent of encephalin was recovered from the brain.

To obtain larger quantities the fresh brain mash was placed in cellophane tubes which were tied at the ends. These 'sausages' were immersed in a volume of $n/100$ HCl, amounting to three times the weight of the brain mash and, with occasional stirring, they were kept in the refrigerator for 24 hours. Then they were transferred into $n/100$ HCl, using half the amount of the first volume.

² This material was originally believed to consist of 'adreno-cortical' compounds, a conception which was later abandoned (38).

After another 24 hours in the refrigerator, the dialyzates were pooled, filtered, placed in bottles with rubber caps (250 cc. in each), frozen and evaporated to dryness. This latter process, which was carried out by the Vermont Red Cross Blood Bank, required about three days.

The dry residue which is rather hygroscopic was kept in the refrigerator in tightly closed flasks. Weighed aliquots were dissolved in water for colorimetric assay and pharmacological testing, this procedure yielding 66-70 per cent of the encephalin contained in the fresh brain.

Since the dialyzate residue proved to contain disturbing by-products, attempts were undertaken to obtain encephalin in greater purity. Various adsorption and subsequent elution procedures were tried with the dry residue of the dialyzate after it had been redissolved in water. Successive adsorptions by $\text{Al}(\text{OH})_3$ at pH 4.0 and 8.5 carried all the encephalin into the alkaline adsorbate, but efforts to set it free again through acidification with HCl yielded only 20-25 per cent.

Adsorptions to nonsoluble adsorbents (kaolin, fuller's earth, carbon) at pH 4.0 and 8.5, and subsequent elution from the adsorbates with HCl eliminated most of the disturbing impurities, but the yields of encephalin too were poor (table 1). Alcohol and ether extracts made from brain mash were likewise unsatisfactory, giving very low yields (1-11 per cent) and eliciting disturbing and sometimes fatal side-effects (deep fall of blood pressure, cardiac standstill).

The following procedure permitted recovery of up to 80 per cent of the encephalin. One gram of the dry residue of the acid dialyzate (see above) is dissolved in 8 cc. of water plus 4 cc. of $\text{Al}(\text{OH})_3$, prepared according to Shaw (23), the pH is adjusted to 4.0 with 1.5 cc. $n/1$ HCl and the suspension is centrifuged at 3000 r.p.m. for about five minutes. To the decanted supernatant fluid one gram of fuller's earth (heated at 500° for 12 hours) is added. The suspension is stirred and centrifuged for about 10 minutes. To the decanted supernatant fluid a 4 per cent NaOH solution (about 1.4 cc.) is added to bring the pH to 8.5, and one gram of fuller's earth. After centrifuging for about 10 minutes, one gram Norit (decolorizing carbon) is added to the decanted supernatant fluid and the suspension is filtered. For storage purposes the filtrate is brought to pH 4.0 by adding $n/1$ HCl (about 0.6 cc.). The encephalin concentration of the filtrates thus obtained ranges between 1 and 3 gamma equ. per cc.

The main disadvantage of this procedure is the fact that the final solution contains potassium salts, amounting to 3-10 mgm. of potassium per gamma equivalent of encephalin. Attempts to remove the potassium by treatment with tartaric acid or chloroplatinic acid were unsuccessful insofar as also most of the encephalin was removed or destroyed.

Finally, the following procedure was adopted which yielded not more than 25 to 30 per cent of the encephalin but with which only 0.3 to 0.5 mgm. of potassium per gamma equivalent of encephalin remained in the ultimate eluate. The first steps are the same as outlined in the preceding paragraph. To the fluid decanted after the first centrifuging, another 4 cc. of $\text{Al}(\text{OH})_3$ are added and about 2 cc. of 4 per cent NaOH, to bring the pH to 8.5. After centrifuging for 5 minutes the supernatant fluid is discarded, 5 cc. of water are added to the sediment, the

sediment is thoroughly stirred up with a glass rod and the suspension is again centrifuged for 5 minutes. After discarding the supernatant fluid, 2 cc. of 3 per cent NaH_2PO_4 are added to the sediment and the latter is again stirred up. Eight drops of $n/1$ HCl are added to bring the pH to 5.0. After 15 minutes the suspension is centrifuged and the supernatant fluid is decanted and placed in a closed flask for cold storage. For injection purposes it can be neutralized with a few drops of saturated sodium bicarbonate solution. (For colorimetric assay (22) 0.25 cc. of the encephalin solution plus 2.2 cc. of water are placed in a test tube and worked up like any specimen having reached the last phase before addition of 0.35 cc. of 4 per cent NaOH . The colorimeter reading must be multiplied with 8 to obtain the concentration of encephalin per cc.)

Pharmacological tests. The effect of encephalin on the blood pressure was tested on atropinized (1 mgm/kgm.) cats under nembutal anesthesia (35–50 mgm/kgm.) and artificial respiration. The completeness of atropinization was usually checked by the failure of the blood pressure to fall after injection of acetylcholine (0.01 mgm/kgm.). The adrenal glands were tied off, the blood pressure was recorded with a mercury manometer from the carotid or femoral artery and intravenous injections were given into the femoral vein.

The dosage of encephalin was calculated from the results of colorimetric determinations carried out in the respective solutions. It was expressed in gamma equivalents and compared with the effects of equal amounts of epinephrine and arterenol (nor-epinephrine, probably identical with sympathin, (29, 30)) under a variety of conditions. The following drugs were used: adrenalin hydrochloride (1:1000), Parke, Davis; dl-arterenol (nor-epinephrine), which was kindly supplied by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute; l-Corbasil (dihydroxy-nor-ephedrine) for which we are indebted to the Farbwerke Höchst, Frankfurt a.M.; tyramine (Burroughs, Wellcome & Co.); atropine sulphate (Parke, Davis); acetylcholine bromide (Eastman Kodak); gynergen (Sandoz); cocaine and dibenamine hydrochloride for which we want to thank Dr. W. Gump of Givaudan-Delawanna Chemical Laboratories.

RESULTS

(1) *Identity of colorimetrically and biologically tested material.* Since the colorimetric and the pharmacological results of this study were largely correlated, it seems advisable to give a brief introductory account of those findings which definitely suggest the identity of the colorimetrically determined chromogenic and the biologically active material under consideration (encephalin).

(a) The quantitative colorimetric and vasopressor effects of equal amounts of brain dialyzate (crude or purified) were practically identical as long as fresh material was used (see section 2, fig. 1). Similar conditions prevailed regarding the effect on the rabbit's intestine and cat's uterus (sections 9, 10).

(b) The recoveries and losses of chromogenic and vasopressor efficiency of encephalin solutions (as tested against epinephrine standards), which occurred during various adsorption procedures and elutions ($\text{Al}(\text{OH})_3$, fullers earth, kaolin), showed a close parallelism (fig. 2). Blood pressure effects could be

approximately predicted from the calculated encephalin concentrations. A short fall of the blood pressure, preceding the characteristic rise which occurred with the injection of encephalin, adsorbed by and again eluted from $\text{Al}(\text{OH})_3$ (fig. 2), was possibly due to contamination with traces of aluminum.

(c) The degree of resistance to destruction through boiling with alkali and through iodine was approximately analogous regarding the chromogenic and vasopressor properties of encephalin (see sections 13, 14).

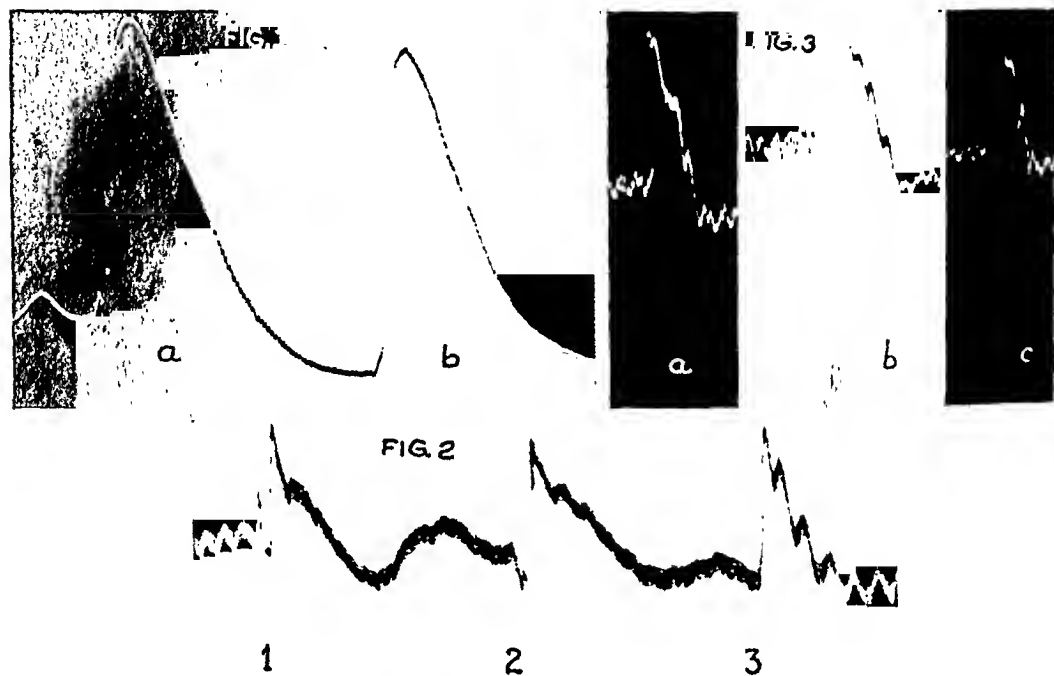


FIG. 1. CAT, atropinized, adrenals tied. a) brain dialyzate (8 gamma equ. encephalin); b) epinephrine (8 gamma).

FIG. 2. CAT, atropinized, adrenals tied. 1) epinephrine (1.5 gamma); 2) cow brain dialyzate (1.5 gamma equ. encephalin, eluted from alkaline adsorbate to $\text{Al}(\text{OH})_3$ through treatment with HCl); 3) cow brain dialyzate (1.5 gamma equ. encephalin, crude).

FIG. 3. CAT, atropinized, adrenals tied. a) arterenol (20 gamma); b) cow brain dialyzate (20 gamma equ. encephalin, crude); c) human brain dialyzate (20 gamma equ. encephalin, crude). Initial falls of blood pressure due to ventricular fibrillation induced by admixture of potassium.

(2) *Blood pressure effects.* The blood pressure response of the cat to intravenous injections of fresh encephalin was practically identical with that elicited by colorimetrically identical, known doses of epinephrine and similar to that elicited by analogous (weight for weight) doses of arterenol (fig. 1, 5a, 11). It reached levels of 200 mm. Hg. (The chromogenic efficiency of arterenol was found to be only about one third of that of epinephrine. A blood pressure effect of arterenol, twice as strong as that of epinephrine as described by von Euler (30), was seen occasionally but not regularly.)

In some instances the encephalin effect differed from the effect of epinephrine

or arterenol by being somewhat weaker or stronger (fig. 3, 5a, 12), occasionally also by forming a different shape of the curve apex (fig. 11). A diminished height of the encephalin blood pressure curves was observed when old material was used. The blood pressure rises after larger doses of crude encephalin were often preceded by a deep fall of the blood pressure (fig. 3). This phenomenon is caused by the toxic effect upon the heart of potassium (contained in the encephalin solutions) which elicits ventricular fibrillation. The vasopressor action of encephalin per se is not affected by the accompanying potassium as demonstrated by the inefficiency of the injection of equal amounts of potassium with equal speed (fig. 4).

Equal amounts of encephalin, obtained from the cortex, the basal ganglia and the white matter of the brain, produced identical effects on the blood pressure.

(3) *Effect of cocaine.* Pretreatment with cocaine (10 mgm/kgm. intramuscularly), while strongly intensifying the vasopressor effects of epinephrine and arterenol in the usual fashion (29, 30), left the effect of encephalin unchanged or even weakened it (fig. 5a, b).

(4) *Effects of ergotamine and dibenamine.* Pretreatment with ergotamine tartrate (4 mgm/kgm.) or with dibenamine hydrochloride (20 mgm/kgm. intraperitoneally) inverted the blood pressure effect of epinephrine but only weakened the effects of arterenol (in confirmation of von Euler's findings, 30) and of encephalin, without inverting them (fig. 6a, b, c).

(5) *Effect of decerebration and pithing.* After decerebration and pithing the vasopressor effect of encephalin was considerably weakened, as compared with that of epinephrine, but still distinctly present.

(6) *Effect on the cat's pupil.* The action of encephalin on the cat's pupil, as compared with that of epinephrine, arterenol and corbasil (dihydroxy-nor-ephedrine), was tested by injection into the carotid artery toward the eye, with the cervical sympathetic cut (fig. 7). While the effects of equipressor doses of arterenol and of corbasil were weaker than that of epinephrine, in agreement with von Euler (30), the effect of equipressor amounts of encephalin was both more intense and more prolonged than that of epinephrine.

(7) *Effect on the isolated frog's heart.* Perfusion of the isolated frog's heart, according to the Straub technique, with insufficiently purified encephalin in Ringer solution at pH 7.0 caused cardiac inhibition in concentrations as low as 0.1 gamma equ./cc., probably due to the presence of excess potassium. Practically potassium-free encephalin was ineffective in frog hearts the response of which to epinephrine was also very poor.

(8) *Effect on the mammalian heart.* Perfusion of the coronary arteries of the isolated rabbit's heart (Langendorff technique, oxygenated glucose Ringer solution at 38°) with encephalin had a stimulating effect, even after the heart had come to a standstill (fig. 8). Electrocardiographic changes, elicited in the cat by the intravenous injection of potassium-free encephalin solutions, resembled those caused by epinephrine or arterenol, regarding deformation of the T waves and displacement of the S-T segment. They will be described in detail elsewhere.

(9) *Effect on the isolated rabbit's intestine.* Rabbit's duodenum, suspended in

a 25 cc. Ringer's solution at 38°, was markedly inhibited by purified encephalin in a dilution of 1:10 million, and slightly in a solution of 1:20 million. The effect was similar to that of equal amounts of epinephrine (fig. 9 a-d). Crude brain dialyzates, on the contrary, produced a stimulating effect.

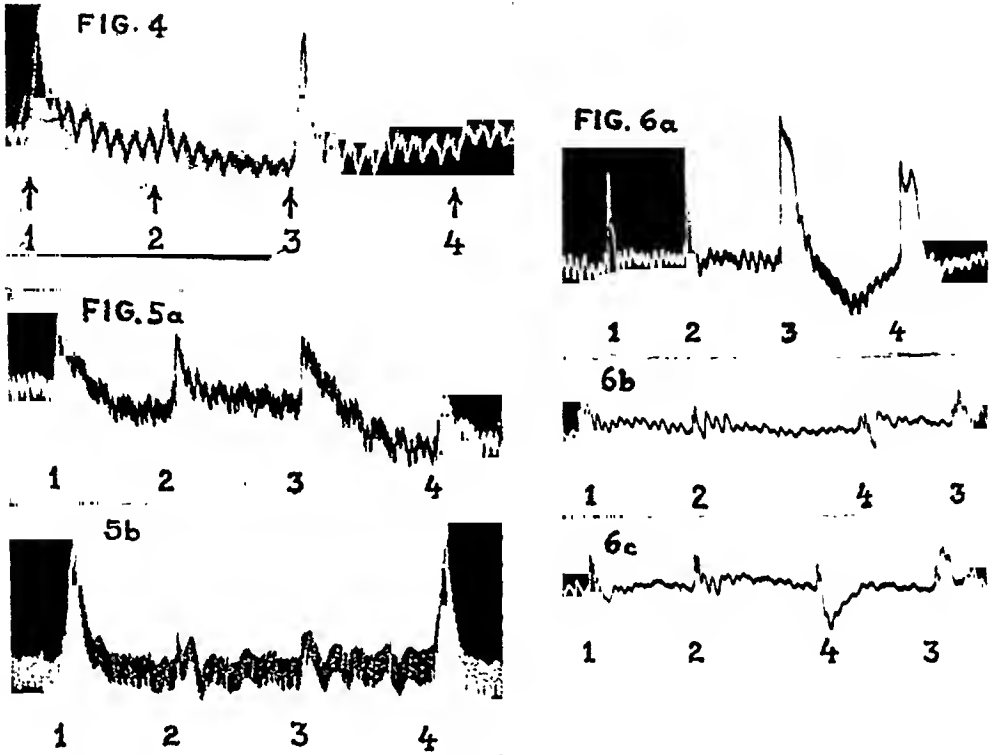


FIG. 4. CAT, atropinized, adrenals tied. Effect on blood pressure of encephalin solution containing 4 mgm. potassium per gamma equ., and of equal amounts of potassium alone (as KCl): 1) encephalin (1 gamma equ.) + 4 mgm. K (0.5 cc. injected in 1 second); 2) 4 mgm. K (0.5 cc. injected in 1 second); 3) encephalin (2 gamma equ.) + 8 mgm. K (1 cc. injected in 2 seconds); 4) 8 mgm. K (1 cc. injected in 2 seconds).

FIG. 5a. CAT, atropinized, adrenals tied. 1) epinephrine (1 gamma); 2) human encephalin (1 gamma equ.); 3) cow encephalin (1 gamma equ.); 4) arterenol (1 gamma). 5b. Same cat 1 hour after 34 mgm. cocaine i.m. Same sequence and dosages as above. Encephalin effect weakened.

FIG. 6a. CAT, atropinized, adrenals tied. 1) human encephalin (5 gamma equ.); 2) calf encephalin (5 gamma equ.); 3) arterenol (5 gamma); 4) epinephrine (5 gamma). 6b. 30 minutes after i.p. inj. of 20 mgm/kgm. dibenamine hydrochloride. Dosages as above. Effect of epinephrine inverted, of encephalin and arterenol only weakened. 6c. 10 minutes later. Each injection 10 gamma (or gamma equ.). Same type of reaction.

(10) *Effect on the isolated nonpregnant cat's uterus.* With a technique analogous to that used for the isolated intestine, encephalin (4 to 8 gamma equ. in a 25 cc. Ringer solution) was found to inhibit the cat's uterus to a somewhat lesser degree than equivalent doses of epinephrine (fig. 10 a-c). The effect of crude brain dialyzates was ambiguous.

(11) *Adsorbability and elutability of encephalin.* The adsorbability of encephalin-

lin by $\text{Al}(\text{OH})_3$, kaolin, fullers' earth and Norit (carbon) was, in general, very much like that of epinephrine and arterenol (table 1) except for some quantitative differences, especially a much lower degree of adsorbability by Norit at pH 8.5, which was made use of in the process of purification of encephalin. Elutability of encephalin from alkaline adsorbates through acidification with HCl was generally rather poor, more so than was the case with epinephrine and arterenol. It was more satisfactory in the presence of NaH_2PO_4 .

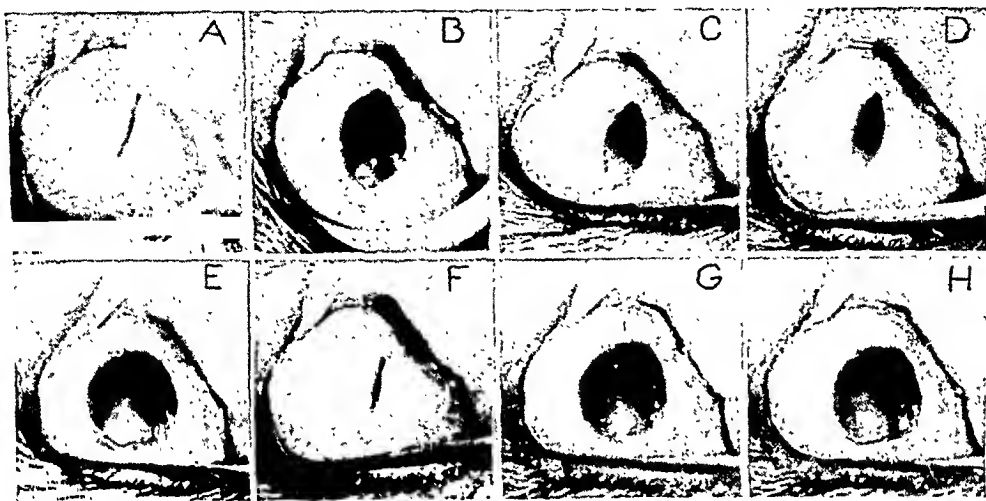
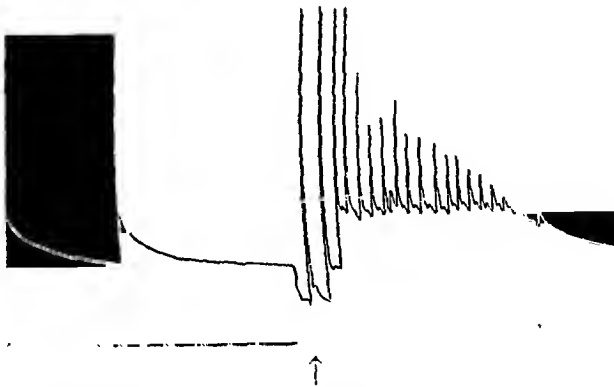


FIG. 7. CAT PUPIL (nembutal, cervical symp. cut), equipressor doses injected. a) control; b) epinephrine (5 gamma); c) nor-epinephrine; d) dihydroxy-nor-ephedrine; e) human encephalin; f) control; g) human encephalin; h) the same as g (1 minute later).

FIG. 8. ISOLATED RABBIT'S HEART perfused with glucose Ringer. After the heart had come to an almost complete standstill, at \uparrow 2 gamma of encephalin (in 2 cc.) were added to the perfusion fluid.



(12) *Stability of encephalin.* When kept in the refrigerator, the brain mash and acid dialyzates (liquid or dry) maintained their chromogenic properties for weeks, with a gradual loss of color intensity, however, amounting to about 10 per cent within one week, to about 65 per cent within 12 weeks. The diminution of vasopressor property seemed to proceed somewhat faster, so that a certain discrepancy between color effect and blood pressure effect used to appear, beginning after about 2 weeks of cold storage. The chromogenic material, present in the cerebrospinal fluid, appeared to be much more stable than that obtained

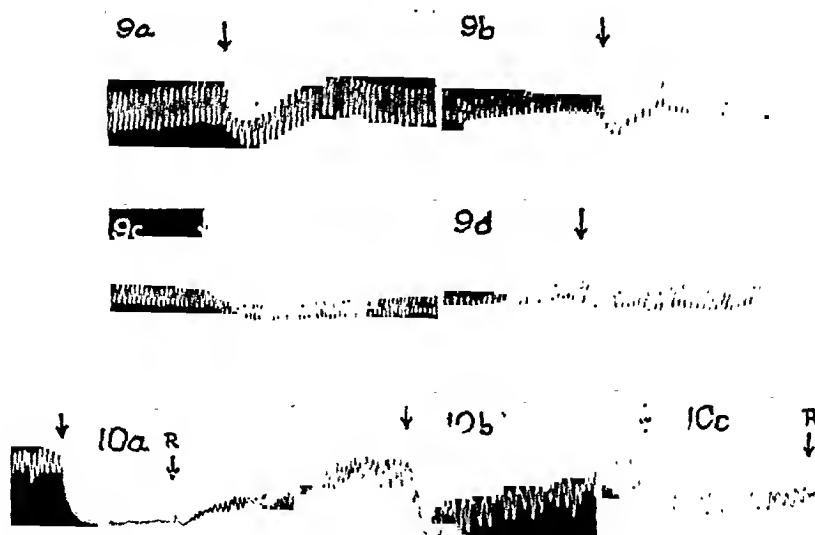


FIG. 9. ISOLATED RABBIT'S INTESTINE. 9a) Epinephrine (1:10 million) eluted from fullers' earth. 9b) cow encephalin (1:10 million) eluted from fullers' earth. 9c) epinephrine (1:20 million) eluted from kaolin. 9d) cow encephalin (1:20 million) eluted from kaolin.

FIG. 10. CAT UTERUS in 25 cc. Ringer. 10a) Epinephrine (4 gamma), R = fresh Ringer; 10b) encephalin (4 gamma equ.); 10c) encephalin (8 gamma equ.), R = fresh Ringer.

TABLE 1

ADSORBENT	EPINEPHRINE	ARTERENOL	ENCEPHALIN
<i>Maximal adsorption observed at pH 4.0</i>			
	%	%	%
Al(OH) ₃	0	0	0
Kaolin.....	73	68	41
Fullers' earth.....	24	0	0
Norit.....	100	100	80
<i>Maximal adsorption observed at pH 8.5</i>			
Al(OH) ₃	100	100	100
Kaolin.....	51	41	17
Fullers' earth.....	0	0	29
Norit.....	100	100	15
<i>Maximal recovery¹ through acid elution with HCl from alkaline adsorbates</i>			
Al(OH) ₃	100	76	25
Kaolin.....	25	23	17
Fullers' earth.....	70	68	29
Norit.....	?	?	8

¹ Expressed in per cent of the original total amounts used in the tests (not of the adsorbed amounts).

from brain dialyzates. It was found quantitatively unchanged after 6 months in the refrigerator and after 8 hours incubation at 37°C.

(13) *Resistance to alkali.* Exposure of epinephrine, arterenol and encephalin (0.2 gamma/cc. each) to intense alkalization (pH 11) at room temperature (30 minutes) did not alter their color effects significantly. When boiled for 15 minutes epinephrine and arterenol lost their chromogenicity completely; encephalin lost 83 per cent of it. Slight alkalization (pH 7.0) and boiling for 30 minutes decolorized epinephrine completely, while arterenol lost only 61 per cent and encephalin 61-77 per cent of their color effects. The diminutions of vasopressor activity roughly paralleled those of chromogenicity.

(14) *Resistance to oxidation through iodine.* Solutions of epinephrine, arterenol and encephalin (25 cc. each, 0.3-1.7 gamma/cc., pH 7.0) were mixed each with 6 drops of $n/10$ I_2 and left standing at room temperature for 5 hours. (In the process of colorimetric analysis the second (alkaline) $Al(OH)_3$ adsorbate had to be carefully washed several times to remove the iodine which is itself chromogenic.) Epinephrine and arterenol were completely decolorized, while the chromogenicity of encephalin solutions was somewhat increased (iodine contamination of the adsorbate?). Correspondingly, the vasopressor effects of epinephrine and arterenol were nearly abolished, while that of encephalin remained almost unchanged (fig. 11). The possibility that encephalin might have been protected against oxidation by iodine through the presence of other accompanying substances in the brain dialyzates was made improbable by the result of mixing epinephrine with a dialyzate and subsequent addition of iodine; epinephrine was again completely destroyed.

(15) *Resistance to ultraviolet irradiation.* Solutions of epinephrine, arterenol and encephalin (20 cc. each, 1 gamma/cc., pH 7.0) were exposed to a therapeutic ultraviolet lamp at a distance of 20 cm. in open Petri dishes, which were cooled by being placed on a wire netting in a water bath with running tap water. Control solutions were also kept in open Petri dishes at the same temperature. Epinephrine and arterenol lost 89 and 90 per cent, respectively, of their chromogenicity within one hour, 100 per cent within 2 hours. The color effect of encephalin on the other hand, remained unchanged. The vasopressor effect of arterenol and epinephrine was completely abolished (the latter in agreement with other observers (34, 35, 36)), that of encephalin was only weakened (fig. 12).

(16) *Color and fluorescence reactions.* Encephalin reduces arsenomolybdic acid, giving rise to a blue color. It has this effect in common with a number of substances, but adsorption by $Al(OH)_3$ at pH 4.0 eliminates some of these, e.g. glutathione, while subsequent adsorption by $Al(OH)_3$ at pH 8.5 separates encephalin, epinephrine, sympathin and related compounds with a benzene ring and two or more free hydroxyl groups in orthoposition from chromogenic contaminants which are not adsorbable at that pH. This type of combined chromogenic and adsorption specificity (see table 1) constitutes the principle upon which the colorimetric procedures for the determination of epinephrine (23, 37) and related compounds (38), including encephalin, are based.

The d.s.r. (denominator of the specific ratio (22, 38)) of encephalin was near

1.0 in almost all of 87 determinations (av. 1.02). This distinguishes it from the d.s.r.'s of epinephrine and arterenol which are nearer 2.0.

Enkephalin differed from epinephrine more fundamentally regarding other color reactions. Addition of one volume of 4 per cent acetic acid and 3 volumes of 4 per cent KIO_3 (39) to one volume of epinephrine solution (100 gamma/cc.) developed a purplish red color while a similar amount of enkephalin turned yellow. FeCl_3 , added to epinephrine, produced a green color which gradually changed into orange. No discoloration took place with enkephalin. The fluorescence re-

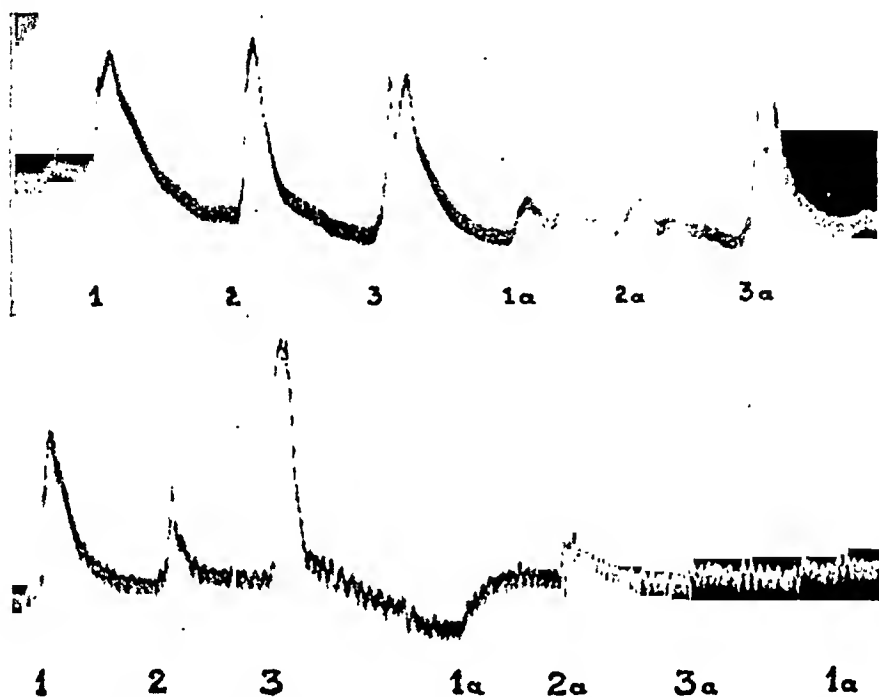


FIG. 11. (upper) CAT, ATROPINIZED, adrenals tied, 0.1 mgm/kgm. gynergen s.c. 1) epinephrine (2.5 gamma); 2) arterenol (2.5 gamma); 3) human enkephalin (2.5 gamma equ.); 1a) epinephrine (2.5) iodine-treated; 2a) arterenol (2.5) iodine-treated; 3a) human brain (2.5) iodine-treated.

FIG. 12 (lower) CAT, ATROPINIZED, adrenals tied. 1) Epinephrine (5 gamma); 2) enkephalin, old (5 gamma equ.); 3) arterenol (5 gamma); 1a) epinephrine (5 gamma) exposed to ultraviolet; 2a) enkephalin (5 gamma equ.) exposed to ultraviolet; 3a) arterenol (5 gamma) exposed to ultraviolet.

action of Gaddum & Schild (52), which gives a strong green fluorescence in alkali-treated epinephrine solutions and a very weak one in alkali-treated arterenol solutions (30), was not present in brain dialyzates. They displayed at first an intense green fluorescence (unspecific ?) which disappeared after alkalization.

(17) *Comparison with tyramine.* Tyramine, which, in contrast to enkephalin, is not chromogenic when brought into contact with arsenomolybdic acid, was injected intravenously into atropinized cats in a dosage several hundred times larger (0.5 mgm.) than the usually effective enkephalin doses. The ensuing ele-

vation of the blood pressure was much more prolonged than the customary effect of encephalin, epinephrine or arterenol; it was much more weakened by cocaine (in agreement with Tainter and Chang (33)) than the effect of encephalin, and pretreatment of tyramine with iodine inverted its effect on the blood pressure, in contrast to the iodine resistance of encephalin (fig. 11).

(18) *Distribution of encephalin in the brain and other parts of the central nervous system.* Chromogenic material of the type of encephalin was found in relatively large amounts in all parts of the central nervous system, including the choroid plexus and the cisternal and lumbar cerebrospinal fluids, but only dialyzates of the hemispheres were tested for biological effects.

The quantitative distribution of encephalin in the brains of 'normal' and diseased humans and of different animal species under various experimental conditions will be discussed elsewhere.

DISCUSSION

The chemical structure of encephalin is unknown. However, the fact that its chromogenic reaction with arsenomolybdic acid, its adsorbability by various adsorbents (table 1) and its pharmacological effects (fig. 1, 5a, 6a, 7-10) resemble those of epinephrine and of arterenol (which is supposed to be identical with sympathin, 30, 42), makes it probable that it is also chemically closely related to these sympathomimetic amines (table 2). That it is not identical with either of them, however, appears evidenced by the following differences: no intensification of the blood pressure effect through cocaine (fig. 5b); no destruction through ultraviolet light (fig. 12); no destruction through iodine (fig. 11); no epinephrine-like color reactions with FeCl_3 and KIO_3 ; no epinephrine-like fluorescence reaction. Regarding inhibition by dibenamine (fig. 6b, c) and resistance to alkali, encephalin corresponds more closely with arterenol than with epinephrine.

The characteristic resistance of encephalin to oxidation by exposure to iodine or to ultraviolet light might be possibly explained by a compound formation of encephalin with lipids, as brain lipids are known to inhibit the oxidation of epinephrine (43), but there are also other sympathomimetic amines (ephedrine, sympatol, isopropylsympatol) which are more or less ultraviolet-resistant by themselves (44).

The nonidentity of encephalin with tyramine was proven by the demonstration of several fundamental discrepancies.

Basic differences between encephalin and pitressin are the following: pitressin is nonchromogenic with arsenomolybdic acid; its blood pressure effect is more prolonged and subject to tachyphylaxis; it stimulates the rabbit's intestine. Other facts which militate against an identity of encephalin with pitressin are the presence of relatively high concentrations of encephalin in the brain cortex and the relatively low concentration of chromogenic material (encephalin ?) in the pituitary gland.

On the other hand, there are some indications that encephalin may be identical with Page's (20) 'C.E.A.' (central excitatory agent) which he found in the

cerebrospinal fluid, also after hypophysectomy. It had the following peculiarities in common with encephalin: vasopressor effect unaltered by cocaine and by

TABLE 2

The data concerning epinephrine and arterenol were largely compiled from the literature (28, 29, 30, 45, 48, 53, 76) but many were also based on original observations which were made in the course of this work and which were in good agreement with those given by other workers.

TYPE OF TEST	EPINEPHRINE	ARTERENOL	ENCEPHALIN
Blood pressure (cat).....	Elevation (++)	Elevation (+++)	Elevation (+++)
Effect of cocaine on bl. pr. reaction.....	Enhanced	Enhanced	Unchanged or weakened
Effect of ergotamine on bl. pr. reaction	Inverted	Weakened	Weakened
Effect of dibenamine on bl. pr. reaction.....	Inverted	Weakened	Weakened
Effect of boiling with alkali on bl. pr. reaction.....	Abolished	Weakened	Weakened
Effect of iodine on bl. pr. reaction.....	Almost abolished	Almost abolished	Unchanged
Effect of ultraviolet irradiation on bl. pr. reaction.....	Abolished	Abolished	Sl. weakened
Effect of pithing on bl. pr. reaction.....	Unchanged	?	Weakened
Isolated mammalian heart.....	Stimulated	?	Stimulated
Electrocardiogram (cat).....	Altered T	Altered T	Altered T
Cat's pupil.....	Dilated (++)	Dilated (+)	Dilated (+++)
Isolated rabbit's intestine.....	Inhibited (++)	Inhibited (+)	Inhibited (+)
Isolated cat's uterus.....	Inhibited (++)	Inhibited (+)	Inhibited (+)
Arsenomolybdic acid test.....	Blue (+++)	Blue (+)	Blue (+++)
Above after boiling with alkali..	No color	Weak blue	Weak blue
Above after treatment with iodine.....	No color	No color	Blue (+++)
Above after irradiation with ultraviolet light.....	No color	No color	Blue (+++)
KIO ₃ test.....	Purplish	?	Yellow
FeCl ₃ test.....	Green	Green	No color
Fluorescence.....	Weak green, with alkali strong	No fl., with alkali weak green	Strong green, with alkali no fl.
Adsorptions and elutions: see table 1			

atropine, weakened by ergotamine. It differed from encephalin by being weakened through repeated injection and by abolition of its vasopressor effect through

pitthng, while the effect of encephalin remained fairly constant with repeated injections and was only weakened by pitthng.

Von Euler's (30) failure to obtain more than very small amounts of a sympathomimetic substance from the brain of a calf is probably due to the fact that encephalin cannot be isolated from the brain in significant quantities by alcoholic extraction, as our own experience suggested.

The presence of what seems to be encephalin in the cerebrospinal fluid can be assumed to be responsible for at least part of the vasopressor properties of this fluid which are usually attributed to secretion of pitressin from the posterior pituitary lobe into the third ventricle. The exact way in which encephalin passes from the brain tissue into the cerebrospinal fluid is not known but its relatively high concentration in the choroid plexus suggests that at least a substantial portion of it is being secreted from there into the ventricular cavities.

Certain neuro-vegetative reactions, analogous to the typical effects of encephalin which occur in animals after stimulation of the hypothalamus (elevation of blood pressure, inhibition of intestinal motility, pupillary dilatation (45, 46, 47)), have been interpreted by some investigators as being due to hypophyseal or adrenal hormonal discharges, but it appears possible that encephalin discharges from the brain via the cerebrospinal fluid into the blood circulation may play a part in the origin and prolonged maintenance of such hypothalamic phenomena.

Morphological indications of a neurosecretory activity of the diencephalic area of the brain have been described by several workers (41, 49, 50, 51).

The 50 per cent lower concentration of what appears to be encephalin in the lumbar fluid, as compared with that in the cisternal fluid, seems to indicate a diffusion of encephalin from the subarachnoidal space into the blood stream. That the difference between the concentrations in the cisternal and lumbar fluid is not due to disintegration of encephalin within the cerebrospinal canal is suggested by the fact that the concentration of chromogenic material present in the cisternal fluid was found unchanged after 8 hours of incubation at 37°C.

Physiological, psychosomatic and psychochemical implications of the presence in the brain of a potent sympathomimetic amine with the characteristics of encephalin are manifold. Some of them are being studied at the present time.

SUMMARY

A sympathomimetic amine was isolated in relatively large quantities from all parts of the human brain and from animal brains.

This substance has some chromogenic and most pharmacodynamic properties in common with epinephrine and with nor-epinephrine supposedly identical with sympathin), but it also differs from both of these in some significant respects. For distinction it has been designated as 'encephalin'.

Apart from donation of drugs, mentioned in the text of this paper, we express appreciation to Dr. O. Loewi (New York) for dialysis equipment, to Prof. U. S. von Euler (Stockholm), Dr. N. B. Dreyer and Dr. Wm. v. B. Robertson, (Burlington, Vt.) for valuable advice and suggestions, to Dr. J. H. Browe (Burlington, Vt.) for taking the photographs of the cat

pupil, to the Department of Pathology of the University of Vermont for the supply of human brains and cerebrospinal fluids and to the Blood Bank of the Vermont Red Cross for freezing and evaporating the brain dialyzates.

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CORONARY SINUS CATHETERIZATION FOR STUDYING CORONARY BLOOD FLOW AND MYOCARDIAL METABOLISM

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Received for publication January 2, 1948

The studies reported in this and the following paper are the result of a combination of two new experimental procedures, each of which was developed and proven practical independently of the other. These are: *a*) a method for collecting coronary venous blood samples by catheterizing the coronary sinus of the dog without opening the chest (1), and *b*) application of the nitrous oxide method, developed by Kety and Schmidt for measuring cerebral blood flow (2) to the dog's coronary circulation (3, 4).

Although incidental catheterization of the coronary venous system in man has been reported by Sosman and Dexter (5), and by Bing and coworkers (6), the procedure apparently had not been employed deliberately, either in animals or man, at the time these experiments were undertaken. The desirability of data obtainable from analysis of coronary venous blood was impressed on one of us (W. T. G.) relative to the study of a case of beri-beri heart disease in man (7). Any biochemical basis for the cardiac weakness here is presumably an interference with pyruvic acid metabolism from thiamine deficiency (8). The level and exchange of pyruvic acid and related metabolites, such as glucose, lactic acid, oxygen and carbon dioxide in coronary venous and arterial blood should be of importance in further study of this condition. Similar data may well offer valuable additional information concerning myocardial failure from other causes. When an opportunity was subsequently presented for exploring the practicability of intentional catheterization of the coronary sinus of the dog, in conjunction with a study of the effects of toxic agents, it was therefore accepted.

Harrison and coworkers (9) were able to cannulate the coronary sinus of intact morphinized dogs using a modified Morawitz brass cannula (10), with a balloon inflated to divert the entire coronary sinus outflow for direct measurement. Coronary sinus outflow, however, is an inconstant fraction of the total coronary blood flow (11, 12). Furthermore, a less traumatic technic was needed, particularly for similar current studies in man (6), preferably a method involving no significant resistance to the coronary venous return. The small flexible Forssman intravenous catheter (13), as modified by Cournand (14, 15), was therefore used in the present studies.

The procedure described in this report was then developed (1), when reports of

recent applications of the nitrous oxide method to the measurement of cerebral blood flow in various diseases (16, 17) suggested that the same method might be combined with the present coronary sinus catheterization technic to permit measurement of coronary blood flow, and hence the oxygen consumption and cardiac efficiency, in the intact dog, preliminary to trial of similar procedures in man (6). The nitrous oxide method avoids the major objection to previous measurements through the coronary sinus which depended upon the absolute volume of sinus outflow, for only *representative samples* of arterial and venous blood are required, such as would be obtained through a small nonoccluding catheter. The coronary blood flow would then be calculated as flow per 100 grams of myocardium drained by the coronary sinus, on the basis of integrated concentrations of nitrous oxide in coronary venous and arterial blood over a 10-minute period of gas inhalation, by an application of the Fick principle (2). The practicability of measuring coronary blood flow by the N_2O method had already been established in the dog by direct cannulation (3), and the indirect method calibrated against simultaneous direct measurements with the bubble flowmeter (4), but no measurements of the coronary blood flow had ever been made in the intact state which the present catheter technique made possible. The special experience and equipment of the two groups were therefore combined in a joint project, and the results in regard to coronary flow and related findings are presented in the second paper of this series (18). This paper deals with the technic and possible hazards of coronary sinus catheterization in the dog and with observations on the biochemical characteristics of coronary venous blood. A third phase of the same joint project, the application of both procedures to similar studies in man, is in progress, in cooperation with Bing and his collaborators at Johns Hopkins Hospital.

Anesthesia and general observations. Pentobarbital sodium, 25 mgm./kgm., was injected slowly intravenously before the procedure, and the dog was maintained in a stage of light anesthesia, with an active lid reflex, a fairly constant pulse and blood pressure and a respiratory rate and pulmonary ventilation adequate to insure a normal arterial oxygen saturation. Arterial hematocrit, hemoglobin content and oxygen saturation (19) were measured frequently throughout each experiment. Although comparable catheterization procedures have been done in unanesthetized animals (20, 21), anesthesia was used in this study chiefly for convenience and to provide a reasonably reproducible and steady status of the circulation (21, 22).

Coronary sinus catheterization. The dog, lightly anesthetized, was placed on the operating-fluoroscopy table and the neck shaved, scrubbed with sponges soaked in alcoholic zephiran solution and draped. Clean but not aseptic technique was followed throughout, with all instruments, catheters and gloves soaked in 1:600 zephiran.

A branch of the external jugular vein was exposed as high as possible in the neck and incised. A curved-tip intravenous catheter (sizes 7-9 F) was then inserted a short distance, and a slow, constant saline drip, containing 20 mgm. of heparin per liter, was connected to prevent clotting of blood in the catheter.

With the dog in the right anterior oblique position, the catheter was passed into

the right auricle under fluoroscopic control. Once inside the superior vena cava, the curved tip was pointed anteromedially to avoid catching it on the ostium of the azygos vein or on the thickened ridge of muscle extending across the posterior auricular wall immediately above the foramen ovale. When the tip reached the inferior portion of the right auricle, near the tricuspid valve, it could be turned posteriorly and easily passed into the inferior vena cava (fig. 1). At this point, with the dog still in the right anterior oblique position, a triangular area of lung with the following boundaries was visible: *a*) the anteromedial border of the inferior vena cava, *b*) the posteroinferior cardiac border, and *c*) the diaphragm. The coronary sinus ostium lies just anteromedial to the superior corner of this triangle, which marks the junction of inferior vena cava and right auricle and which lies posteroinferior to the tricuspid valve. The catheter was now slowly withdrawn from the inferior cava to a point just inside the right auricle. If the tip was then turned and shifted slightly anteromedially, it pointed directly toward the coronary sinus ostium.

After one or two gentle thrusts, the catheter usually rounded a sharp initial bend (fig. 1A) and entered the sinus. The tip often passed further into the sinus, superiorly and to the left along the posterior auriculo-ventricular groove (fig. 1B) and sometimes beyond one or more delicate valves into the great cardiac vein (fig. 1C). Occasionally the ostium of the middle cardiac vein (fig. 5), located just inside the ostium of the coronary sinus (fig. 4), or more rarely, a posterior vein of the left ventricle (fig. 6), was accidentally catheterized. These veins pass along the posteroinferior septal surface toward the apex, anastomosing with each other and with the anterior descending branch of the great cardiac vein, as shown by diodrast injection in figure 6. By preference, however, only the proximal coronary sinus itself now is catheterized (fig. 1A and 1B), as discussed below, because of the dangers and technical invalidity of coronary venous obstruction.

The advantage of the right anterior oblique position for visualization of the path of the catheter into the coronary sinus is illustrated in figure 2B. In this view, a sharp bend was seen in the course of the catheter as it entered the sinus. The curve appeared foreshortened and could be seen in full length only in a more posteroanterior position (fig. 2C and 2D). In the latter view, however, the pathway was almost superimposable upon the path of a catheter entering the right ventricle and pulmonary artery, and it became extremely difficult to tell exactly where the catheter was. The chief difference between the two pathways was the more posterior orientation of the coronary sinus, which became distinct as the animal was rotated to the oblique position. In this view, furthermore, the closest and most prominent landmark to the position of the coronary sinus ostium, namely the lung triangle and inferior vena cava, were well visualized. If the dog was turned too far toward the right lateral position (fig. 2A), the pathway into the coronary sinus became difficult to visualize because of a loss of three-dimensional perspective.

Coronary venograms. Diodrast (3,5 diiodo-4-pyridone-N-acetic acid and diethanolamine) has been injected forcibly through the catheter against the

stream of coronary sinus flow, and, as shown in figure 4A and 6, a retrograde coronary venogram obtained. If the catheter obstructed the vein, as in figure

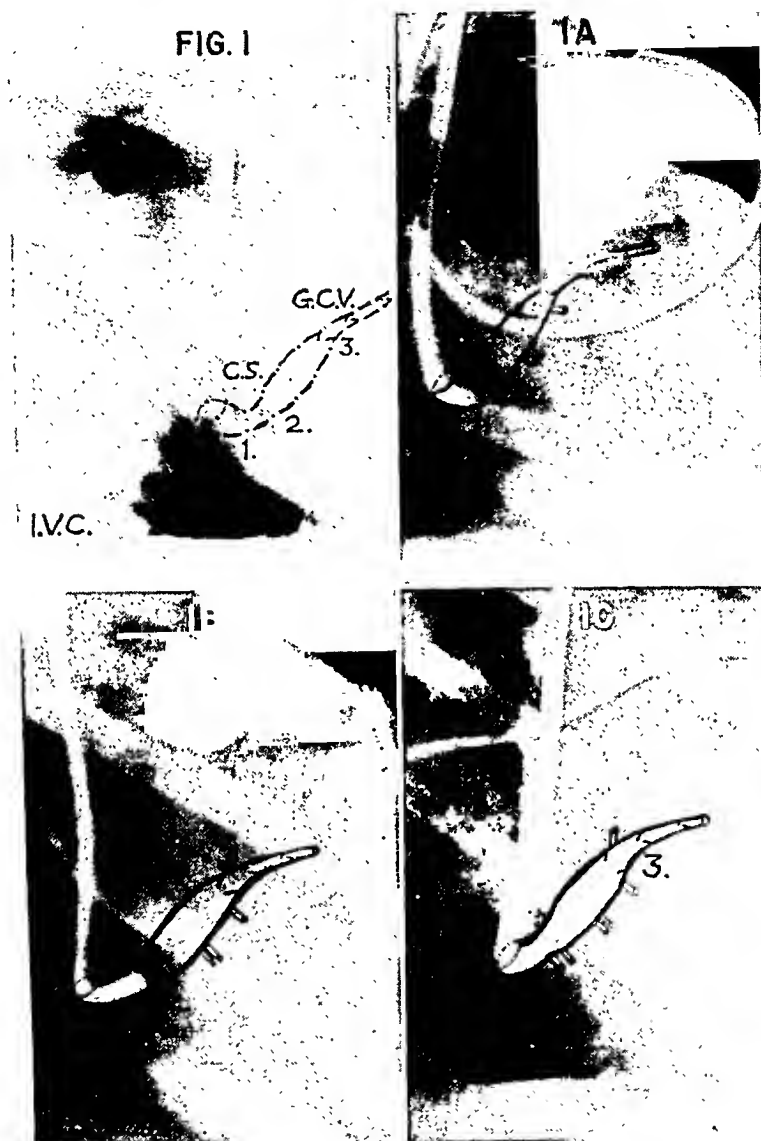


FIG. 1. CATHETER INSERTED INTO THE INFERIOR VENA CAVA (I.V.C.), illustrating the lung triangle (L.T.) and its relationship to the coronary sinus (C.S.) and great cardiac vein (G.C.V.) in the right anterior oblique view.

FIG. 1A. CATHETER INSERTED ONE TO 1.5 CM. INTO CORONARY SINUS. *Position 1.*

FIG. 1B. CATHETER INSERTED TWO TO 2.5 CM., JUST BEYOND THE SHARP BEND IN THE SINUS. *Position 2.*

FIG. 1C. CATHETER INSERTED INTO GREAT CARDIAC VEIN, MORE THAN THREE CM. BEYOND THE SINUS OSTIUM. *Position 3.*

5 and 6, diodrast could be easily made to fill most of the veins draining into the coronary sinus, due to the multiple veno-venous anastomoses. If the catheter lay freely in the coronary sinus, the diodrast was carried back along the shaft

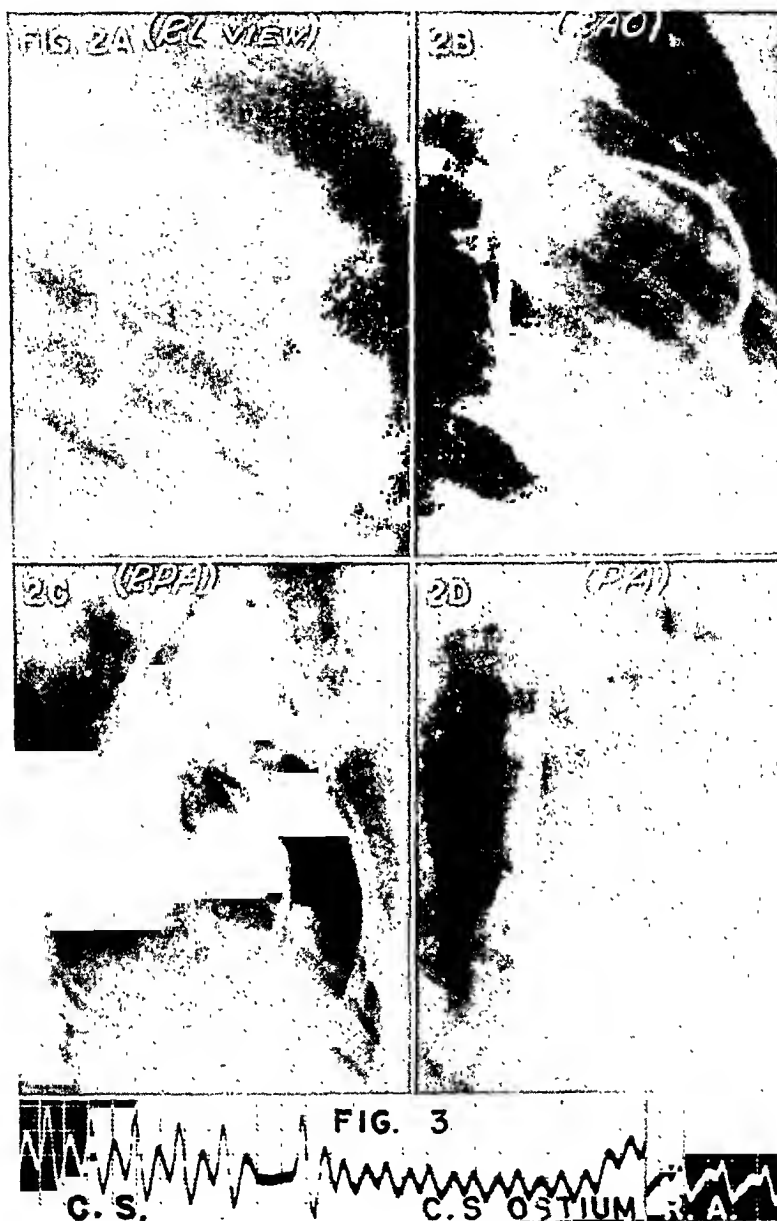


FIG. 2A-D. COMPARISON OF RIGHT LATERAL (RL), right anterior oblique (RAO), right posteroanterior (RPA) and posteroanterior (PA) in the same dog. The large catheter is inserted 4 to 5 cm. into the coronary sinus, the small catheter into the pulmonary artery. The advantage of the RAO view for distinctive visualization of the catheter in the coronary sinus is illustrated.

FIG. 3. PULSE PRESSURE CURVES, slightly damped, during withdrawal of the catheter from the coronary sinus, recorded through a Statham strain gauge on a Sanborn cardiette amplifier. Scale: one cm. excursion = 6 mm. Hg pressure. Systolic pulse pressures: coronary sinus (position 3), 11 mm. Hg; coronary sinus ostium (position 1), 5 mm. Hg; right auricle, 5 mm. Hg. Autopsy in this case showed that the catheter inserted to position 3, where the elevated pressure was noted as illustrated, probably caused a significant degree of coronary venous obstruction.

of the catheter in a visible stream with the normal coronary sinus flow until rapidly dispersed into the auricle at the coronary sinus ostium. The use of diodrast has helped to establish the orientation of the coronary sinus and coronary venous system as seen fluoroscopically, as well as the in vivo relationship of the coronary sinus ostium to the tricuspid valve (fig. 4A). Forcible injection is hazardous, however, since the dye may be forced into the tissues and cause local myocardial necrosis (fig. 5).

Design of coronary sinus catheters. The ordinary curved tip intravenous catheter presented the following drawbacks when used for catheterization of the coronary sinus:

a) Although fluid could always be freely injected through the catheter into the sinus, withdrawal of blood samples was often difficult or impossible. It is likely that the wall of the sinus, or a valve leaflet in the sinus or great cardiac vein, was drawn against the single catheter opening when suction was applied. Often blood could only be withdrawn either at the height of inspiration, when the descent of the diaphragm altered the position of the heart and of the catheter tip in the sinus, or during systole when the coronary venous pressure rose. This difficulty has been more serious, both in dogs and in man, when a small catheter (#6 or #7 F) has been used.

b) A small catheter (#6 or #7 F) would be most desirable for avoiding coronary venous obstruction. Such small catheters, however, were much harder to insert into the sinus than the larger sizes, because they tend to buckle in the auricle without passing around the sharp bend in the sinus. The ideal catheter then should have a small flexible tip for the part actually within the sinus and a stiff, nonbuckling shaft. This would give the best control on insertion and the least obstruction and trauma to the coronary sinus. A larger, stiffer catheter may be actually less traumatic than a smaller one which is less easily controlled because of buckling (23). A stylet, inserted to within 3–4 cm. of the catheter tip, has been used in many instances with some improvement in control, but the use of a stylet creates other technical difficulties without solving the problem of obstruction of the catheter opening.

A modified catheter has largely solved these difficulties. The shaft tapers from a stiff #8F to a more flexible #6 or #7F between 4 and 6 cm. from the tip, and there are two additional side eyes 2 mm. back from the usual terminal eye. The side eyes tend to break any possible suction on a vessel wall or valve leaflet as a sample is withdrawn. The tendency to buckle is minimal with the larger shaft, while the smaller tip causes less trauma and obstruction within the coronary sinus. (These modified catheters are available on special order from the U. S. Catheter and Instrument Co., Glens Falls, N. Y.).

Care of catheters. The original stiffness of the intravenous catheter shaft is essential to its controlled manipulation and should be restored after use by drying the catheter in an oven at approximately 90°C. for one to two hours. An inlying stylet, curved at the tip as desired, helps to keep the catheter in shape while drying. The stylet is removed after the catheter cools.

Clotting in the tip, particularly in catheters with multiple openings, may be

avoided by soaking the tip in heparin solution for 30 minutes before use. The hydrophilic plastic enamel of the catheter will take up a trace of the solution. Finally, just before use, the entire lumen is flushed with the same heparin solution. Clotting in the tip may also be prevented by coating it with Silicone (dri film #9987, available from General Electric Co., Schenectady, N. Y.).

Catheterization of the pulmonary artery. For sampling mixed venous blood, a #6 or #7 F, curved-tip intravenous catheter was inserted into the same vein used for the coronary sinus catheter. By clamping the opposite sides of the vein walls between the two catheters, retrograde bleeding could largely be prevented. A saline drip, containing 10 mgm. of heparin per liter, was connected, and the catheter was then inserted into the pulmonary artery by the technique of Kinney, Haynes and Dexter (24). The tip was left far enough out into the lung field to be sure of its position, but not so far out, as Dexter and coworkers have shown can be done (23), as to draw fully oxygenated blood from the pulmonary capillaries or possible precapillary arteriovenous anastomoses.

Catheterization of the femoral artery. The femoral artery was exposed low in the femoral canal, and a #6 or #7 catheter inserted through a small incision in the artery, passed up into the aorta and tied in place. The catheter was connected to a mercury manometer for pressure recordings except during sampling.

Sampling and analytical methods. Blood samples were drawn simultaneously from the femoral and pulmonary arteries and coronary sinus through manifold systems (25) and kept ice-cold until analyzed. *Blood oxygen content* was measured by the method of Roughton and Scholander (26), checked against the method of Van Slyke and Neill (27). The latter method was also used for *carbon dioxide content* (27). *Coronary blood flow* was determined by the nitrous oxide method of Kety and Schmidt (2, 25), as applied to the coronary circulation in the accompanying report (18). Glucose samples were analyzed by Nelson's method (28), using Somogyi's copper reagent (29), pyruvic acid by the method of Friedemann and Haugen (30) and lactic acid as outlined by Barker and Summerson (31).

RESULTS

The coronary sinuses of 45 dogs, weighing 15 to 40 kgm., have been catheterized 68 times by this technique. There have been three failures, two of which were in dogs under 15 kgm. The procedure has permitted repeated observations of coronary blood flow and myocardial metabolism in the same dogs as often as seven times over a four-month period, as illustrated in the protocol of dog #7 in figure 4 and table 1. The coronary blood flow averaged 71 cc. per 100 grams of left ventricular myocardium per minute, with a myocardial oxygen consumption averaging 9.7 cc. per 100 grams per minute. In current experiments, these observations together with an estimation of cardiac work permit the calculation of the overall mechanical efficiency of the heart.

A. *Evidence of successful coronary sinus catheterization*, (in addition to fluoroscopy). 1. The dark color of coronary sinus blood samples, corresponding to a very low oxygen saturation, clearly distinguished it even on inspection from

other venous blood samples. The oxygen content of coronary venous blood averaged 4.1 volumes per cent (approximately 22 per cent saturated), as compared with a mixed venous content of 13.0 and an arterial content of 17.2. The mean coronary arteriovenous oxygen difference was thus approximately three times the total systemic A-V difference, (table 2).

2. A typical pulse pressure pattern (fig. 3) was seen when the catheter was inserted well into the coronary sinus or great cardiac vein, correlated with a marked pulsation of the blood as withdrawn or pulsation of the intravenous drip. This finding was considered to indicate at least partial coronary venous obstruction, confirmed by the elevated pulse pressure of 9–20 mm. Hg compared with a right auricular pressure of 3 to 5 mm. Pulse pressures on insertion of a small catheter a similar distance, or on withdrawal of a larger catheter to position

TABLE 1. RESULTS OF REPEATED CORONARY SINUS CATHETERIZATIONS OF THE SAME NORMAL DOG

Dog #7, male, body weight 31 kgm., heart wt. 240 gram

(See protocol, fig. 4)

DATE, 1947	17 JUNE		8 JULY		29 JULY	19 AUG.		AVE.
Experiment No.....	1	2	1	2	1	1	2	
Coronary Blood Flow, cc./100 gm./min.....	65	58	91	77	56	79	71	71
Cardiac O ₂ Consumption, cc./100 Gm./min.....	8.7	8.2	12.4	10.5	8.3	9.8	10.1	9.7
Arterial O ₂ Content, vols. %.....	15.4	16.3	17.0	16.6	18.5	16.5	18.2	16.9
Coronary Venous O ₂ , vols. %.....	2.0	2.2	3.3	2.9	3.5	4.1	3.7	3.1
Mean Arterial B. P., mm. Hg.....	135	147	142	146	142	142	115	138
Cardiac Rate Per Min.....	155	160	135	130	140	160	156	148

1, just inside the coronary sinus ostium, were no more than 2 to 3 mm. higher than right auricular pressures (fig. 1A), with minimal visible evidence of pulsation in the intravenous drip.

B. *Biochemical characteristics of coronary venous blood.* Analysis of coronary venous blood clearly distinguished it from mixed venous or arterial blood, (table 2). No significant difference in these values was found when the catheter was inserted deeply into the sinus or great cardiac vein instead of only one to 2 cm. inside the ostium. The consistently high coronary arteriovenous differences of oxygen, lactate and pyruvate, considering the rate of coronary blood flow, indicate an extremely high rate of myocardial utilization of these metabolites in the normal, intact, lightly anesthetized dog. The rate of carbon dioxide production is correspondingly high. This confirms previous findings of many investigators who have used open chest and heart-lung preparations, (32–34).

Glucose was removed by the heart in relatively small amounts, often not at all, and highly inconsistently as shown by the large standard error in table 2.

Because of the interest in the constancy of the lactate/pyruvate ratio the relationship has been calculated on a small series of arterial bloods (n = 11)

yielding a correlation coefficient of 0.88, and a mean L/P ratio of 8. No significant difference in the ratio was found in coronary venous blood.

The cardiac lactic and pyruvic acid utilization alone could account for 40 to 60 per cent of the total cardiac oxygen utilization in these experiments, assuming eventual complete oxidation of these metabolites to carbon dioxide and water.

C. *The anatomy of the coronary sinus venous system of the dog.* Twenty-eight anatomical dissections, correlated with diodrast injections in vivo, have helped to clarify the position of the catheter with relation to the coronary sinus venous system as seen fluoroscopically (figs. 4-6).

The coronary sinus lies in the posterior auriculoventricular groove and is surrounded by epicardial fat. The sinus takes origin embryologically from the

TABLE 2. BIOCHEMICAL CHARACTERISTICS OF CORONARY SINUS BLOOD

	n	MIXED VENOUS	ARTERIAL	CORONARY SINUS	MEAN ARTERIOVENOUS DIFFERENCE OF SIMULTANEOUS OBSERVATIONS ¹	
					Systemic	Coronary
		mean $\pm \sigma_m$	mean $\pm \sigma_m$	mean $\pm \sigma_m$	mean $\pm \sigma_m$	mean $\pm \sigma_m$
Oxygen, vols. %...	21	13.0 \pm .50	17.2 \pm .42	4.1 \pm .19	+4.2 \pm .39	+13.0 \pm 5.4
Carbon diox- ide, vols. %	10	35.9 \pm 1.20	31.8 \pm 1.20	44.5 \pm 1.26	-4.1 \pm .19	-12.6 \pm .71
Lactic acid, mgm. %...	20	15.8 \pm 2.1	15.3 \pm 2.0	9.0 \pm 1.1	-.50 \pm .30	+6.5 \pm .99
Pyruvic acid, mgm. %...	14	1.93 \pm .14	1.99 \pm .17	1.07 \pm .10	+.07 \pm .09	+.93 \pm .15
Glucose, mgm. %...	16	78.0 \pm 3.3	79.3 \pm 4.1	75.3 \pm 3.6	+1.3 \pm 2.5	+4.0 \pm 2.6

¹ The difference between each pair of simultaneously drawn samples was obtained and the average of these differences determined.

junction of the great cardiac vein with the oblique vein of Marshall. The latter is the remnant of the fetal left superior vena cava and is often difficult to identify in the adult dog. We therefore measured the length of the coronary sinus to the first valve, which was usually bicuspid and well formed and which often coincided with a sharp change in the color of the wall from dark to light. This color change is caused by a marked thinning of the muscularis. In the absence of a demonstrable valve, the point of color change alone is used as a guide. With these criteria the length of the coronary sinus measures 2 to 5 cm. and is only roughly related to the size of the heart. The catheter may appear fluoroscopically to be inserted somewhat more deeply, since it follows and probably distends the outer wall of the coronary sinus as it curves around the heart. Beyond the first valve there are often one to four single or bicuspid valves in rapid succession (fig. 1). The auricular ostium of the coronary sinus, however, is not guarded by the well-defined Thebesian valve usually found in man.

The middle cardiac vein enters the sinus not more than 0.5 cm. from the

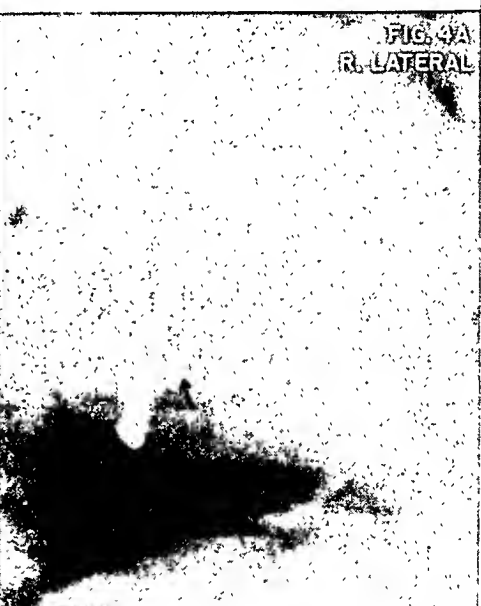
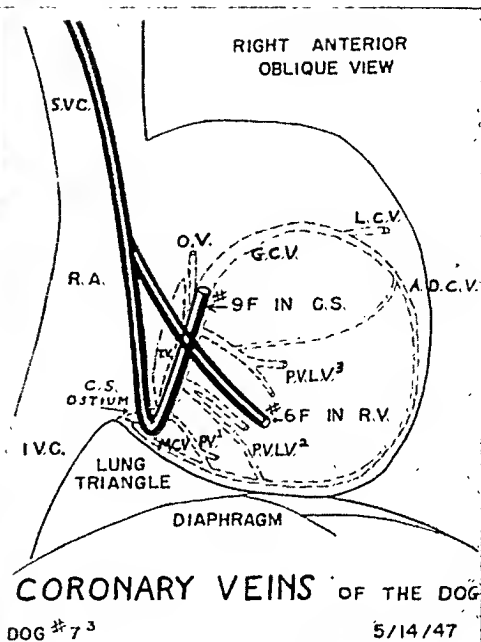


FIG. 4. THE ANATOMY OF THE CORONARY SINUS VENOUS SYSTEM in relation to a large #9 catheter, inserted three to four cm. into the coronary sinus (C.S.), with a small #6 catheter passing through the tricuspid valve (— — — —) into the right ventricle (R.V.). The coronary sinus venous system, as found at autopsy, is sketched as follows: middle cardiac vein, M.C.V.; posterior veins of the left ventricle, P.V.¹, P.V.L.V.², P.V.L.V.³; oblique vein of Marshall; O.V.; great cardiac vein, G.C.V.; left circumflex vein, L.C.V.; anterior descending coronary vein, A.D.C.V. The extensive veno-venous anastomoses between the major veins draining the left ventricular myocardium are illustrated.

Metabolic observations. See table 1.

Autopsy. This dog was catheterized seven times in five months. The catheter was in the sinus a total of 16 hours, and there was usually a second catheter in the pulmonary artery or right ventricle. Autopsy seven weeks after the last procedure showed a normal heart except for slight subendocardial fibrosis in the right auricle and thickening of the medial tricuspid valve leaflet.

FIG. 4A. DIODRAST INJECTION OUTLINING THE GREAT CARDIAC VEIN and its branches. Arrow points to diodrast flowing with the coronary venous return from the coronary sinus ostium through the tricuspid valve.

FIG. 5. CATHETER OBSTRUCTING THE MIDDLE CARDIAC VEIN. The shadow of diodrast in the tissues illustrates the danger of forcible injections when the vein is obstructed.

auricle. It drains the posterior septal area and often a small rim of right ventricle and is often too small to admit a catheter tip. Two to four major posterior left ventricular veins open into the sinus between one and 4 cm. from its auricular ostium. These veins vary in size and number and often have multiple openings into the coronary sinus. There are usually abundant veno-venous anastomoses, not only among the middle and posterior veins, but also between one or more of these veins and the anterior descending vein near the apex. The veno-venous anastomoses are best demonstrated by diodrast injection in vivo, (figs. 4A-6). Beyond the last posterior vein for a distance of 1.25 to 3 cm., there are no significant venous junctions.

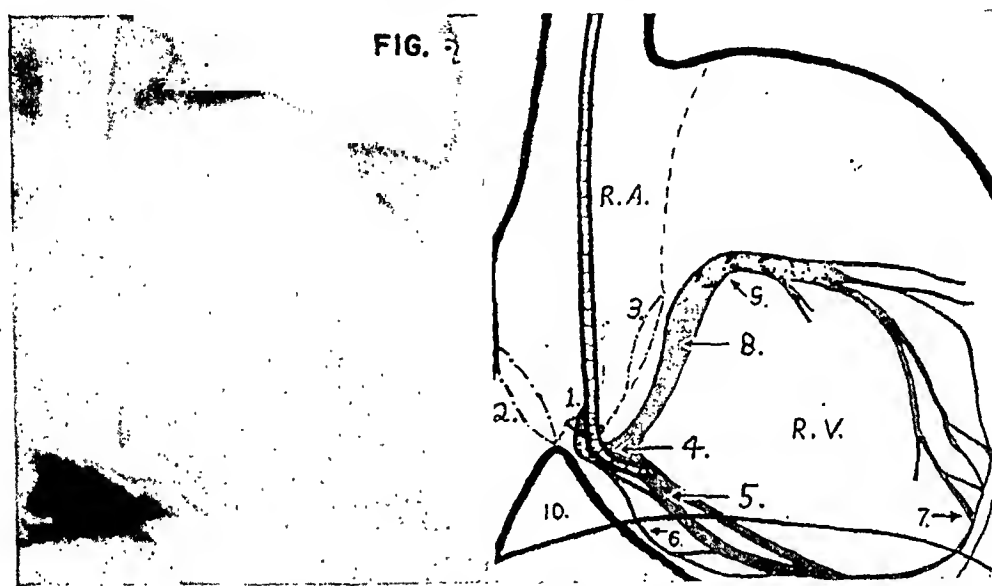


FIG. 6. CATHETER OBSTRUCTING THE FIRST POSTERIOR VEIN of the left ventricle. Diodrast injection outlines many of the veins draining into the coronary sinus through veno-venous anastomoses. 1. coronary sinus ostium; 2. inferior vena cava; 3. tricuspid valve; 4. coronary sinus position 1; 5. first posterior vein of the left ventricle; 6. middle cardiac vein; 7. apical veno-venous anastomoses; 8. coronary sinus position 3; 9. great cardiac vein with valves illustrated; 10. lung triangle.

There is a constriction of the sinus at the first valve and a sharp decrease in caliber of the great cardiac vein as it swings down the anterior longitudinal sulcus. This vein drains the anterolateral surface of the left ventricular myocardium and also receives branches from the left auricle.

In summary, diodrast venograms and autopsy studies indicate that a nonoccluding catheter, inserted only one to two cm. inside the auricular ostium of the coronary sinus (position 1 or 2, fig. 1A or 1B), should collect representative samples of coronary venous blood draining almost entirely from the left ventricular myocardium. If this position of the catheter tip allowed withdrawal of blood from the middle cardiac vein, a significant proportion of the right ventricular myocardial flow might be obtained. Gregg has emphasized, however, that the anterior cardiac veins, which do not drain into the coronary sinus, are the

major drainage channels of the right ventricle (11). The physiological role of the Thebesian veins is still disputed, (11, 12). Because of the sharp decrease in venous caliber at the opening of the great cardiac vein, however, varying degrees of venous obstruction may occur when the catheter is inserted too far, as already noted *in vivo* from pulse pressure recordings.

D. *Hazards of coronary sinus catheterization and other catheterization procedures in dogs.* In 48 survival experiments, recovery was uniformly prompt, without any clinical complications attributable to catheterization. Postoperative care included one dose of penicillin in beeswax and oil, 300,000 units *i.m.*, 0.4 grams of ferrous sulfate daily and exercise with return to routine diet as early as 12 hours after the experiment.

Autopsy studies, however, often showed endocardial damage when the dogs were sacrificed following catheterization of not only the coronary sinus but also the right auricle and pulmonary artery, (35). A more controlled study is now in progress, but the findings incidental to the development of coronary sinus catheterization technique may be summarized as follows:

In a recent series of 28 dogs, autopsied within 10 days after catheterization, only three dogs were free of lesions at autopsy. Six had minimal lesions consisting of small subendocardial hemorrhages and tiny mural thrombi. The remainder had moderate to severe lesions of varying sizes, consisting of mural thrombi and subendocardial hemorrhages which sometimes extended well into the myocardium. These lesions occurred along the course of insertion of the catheter, including the tricuspid and pulmonary valves after catheterization of the pulmonary artery.

Six autopsies three to six weeks following catheterization showed small patches of minimal subendocardial fibrosis in three cases; one of these showed a small, well-organized thrombus in the superior vena cava. There was one case of marked fibrosis of the medial tricuspid valve leaflet, possibly unrelated to catheterization. There were no lesions in two cases.

In these dogs, varying degrees of difficulty in catheterization were encountered. Catheters ranging from sizes 6 to 10 were used, usually #7 or 8. The catheter was left in place from zero to five hours. Aseptic technique, omission of heparin administration and use of a small catheter did not consistently eliminate the occurrence of endocardial lesions, whether the catheter was inserted into the coronary sinus, pulmonary artery or only the right auricle.

This series was not well enough controlled to draw definite conclusions, but endocardial lesions were usually minimal when extreme care was taken to avoid trauma on insertion and when the catheter was left in place no more than one and one half hours.

Deep catheterization of the coronary sinus, however, to position 3 or beyond (fig. 1C), often caused lesions peculiar to this procedure alone. There were three cases of gross myocardial hemorrhage in areas drained by the catheterized vein, particularly in the mitral valve and apical areas. In two additional cases of deep prolonged insertion, there was a well organized thrombus occluding the great cardiac vein. These lesions have been consistently avoided in 16 recent

cases using definite precautions, including gentle insertion of a #7 catheter only one to 2 cm. inside the sinus.

Electrocardiographic studies. Limb and precordial electrocardiograms were taken before, during and after coronary sinus catheterization in 34 experiments. In three out of 11 dogs in which the catheter was inserted into the great cardiac vein, (position 3 or beyond, fig. 1C), T-wave inversions and S-T segment elevations were noted following the procedure, in one or more leads, which suggested pericardial (or epicardial) irritation. Such changes were associated at subsequent autopsy in one case with thrombotic occlusion of the great cardiac vein and in another with hemorrhage of the myocardium beneath the epicardium, in areas drained by the catheterized vein. No electrocardiographic changes were noted following 23 experiments in which the catheter was inserted only one to 2 cm. inside the coronary sinus.

TABLE 3. CONTROL VALUES OBTAINED BY REPEATED CATHETERIZATIONS OF THE SAME ANIMAL
(male dog—weight 31 kgm.)

DATE		CORONARY FLOW	O ₂ CONSUMP.	ART. O ₂	VEIN O ₂	MABP	CARDIAC RATE
		cc/100G/min.	cc/100G/min.	vol. %	vol. %	mm Hg	
6/17/47	#1	64.5	8.65	15.4	2.0	135	155
	#2	58.0	8.18	16.3	2.2	147	160
7/ 8/47	#1	90.5	12.4	17.0	3.3	142	135
	#2	77.0	10.5	16.6	2.9	146	130
7/29/47	#1	55.5	8.34	18.5	3.5	142	140
8/19/47	#1	79.0	9.80	16.5	4.1	142	160
	#2	71.0	10.12	18.0	3.7	116	156
Average.....		70.8	9.71	16.9	3.1	138	148

Continuous recordings on Lead II during insertion of the catheter into the coronary sinus, among 37 experiments, showed only occasional isolated nodal extrasystoles in 32 cases, and one case of transient nodal tachycardia lasting 20 seconds. Insertion of the catheter through the tricuspid valve, however, invariably produced very rapid bursts of ventricular and nodal extrasystoles. Similar extrasystoles were sometimes seen when the catheter tip hit the right ventricular wall below the pulmonary conus.

DISCUSSION

Endocardial damage from catheterization of the heart by the present technique has not been reported previously in dogs or man, except for one case presented by Johnson (36). This patient had congenital heart disease with cyanosis and polycythemia, and at autopsy showed multiple thrombi along the course of insertion of the catheter one month following the procedure. In several large series, however, covering over a thousand catheterizations by several investigators, and including numerous autopsied cases, there has been no apparent damage

to the heart from catheterization procedures in man, (6, 15, 23). The fact that a catheter often causes endocardial lesions in dogs, but apparently not in man, may be related to differences in technique, or more likely, to species differences in the response to injury. For example, the general tendency to blood clotting and postoperative intravascular thrombosis has been commonly observed to be greater in dogs than in man (37). However, thrombi in dogs were seldom present less than 12 hours after catheterization, or more than three weeks after catheterization, so that healing apparently occurred within three weeks. Lesions in man resulting from catheterization thus might not be found unless autopsy were performed within a comparable period. It seems unlikely that insertion of the catheter through the external jugular vein can be the determining factor, or that this approach is much more traumatic than the brachial approach used in man. Anesthesia may in some way influence the development of lesions. Tachycardia under nembutal anesthesia, and the poor fixation of the heart in the dog's mediastinum, may increase the friction between catheter and endocardium. The size of these hearts, however (90–240 grams), is well within the range of the hearts of children and some adults.

In conclusion, deep insertion of a catheter three cm. or more into the coronary sinus or great cardiac vein in dogs often appears to be unduly traumatic and undesirable because of coronary venous obstruction. This conclusion is based upon a correlation of anatomical and pathological studies with electrocardiographic and pressure recordings *in vivo*. A recent series of experiments, presented in the accompanying report (18), have yielded satisfactory coronary flow measurements with the catheter inserted only one to 2 cm. inside the sinus ostium (fig. 1A or 1B) with minimal damage from the procedure and with no evidence of contamination of blood samples with auricular blood. Therefore, definite precautions, including gentle insertion of a small catheter only a short distance inside the coronary sinus ostium, may well be advisable in applying this technic to similar studies in man. With these precautions in the dog, however, coronary sinus catheterization appears to be no more traumatic than catheterization of the pulmonary artery.¹

SUMMARY

1. A technic of coronary sinus catheterization in intact dogs has been presented. Modifications in the catheter design and the optimal position of the dog for fluoroscopic control of insertion have been discussed. The procedure has permitted repeated observations of coronary blood flow and myocardial metabolism in the same dogs over a period of months.

2. A high rate of cardiac utilization of oxygen, lactate and pyruvate, with a correspondingly high carbon dioxide production, were consistently found in the normal dog. Glucose utilization was relatively lower and highly inconstant.

¹ The occurrence of endocardial lesions in dogs following catheterization of the pulmonary artery has been confirmed recently by Hellems, Haynes, Fanger and Dexter (38). The lesions were similar, but occurred with less frequency and severity than in the present series.

3. Although technical refinements have minimized the occurrence and size of lesions, it should be realized that endocardial damage cannot yet be entirely avoided in dogs following catheterization of not only the coronary sinus but also the pulmonary artery.

4. Peculiar to coronary sinus catheterization, however, were the coronary venous thromboses or myocardial hemorrhages which sometimes followed prolonged insertion of a catheter far into the coronary sinus or a cardiac vein. The elevated pulse pressure, 9 to 20 mm. Hg, found on deep insertion, indicated a significant degree of coronary venous obstruction by the catheter.

5. Coronary venous and myocardial damage were avoided by definite precautions, including gentle insertion of a small catheter only one to two cm. in side the coronary sinus. In this position there was no evident admixture of coronary blood samples with auricular blood, evidence of trauma to the auricle or coronary sinus ostium was minimal and pulse pressures were the same or only slightly higher than in the auricle, indicating that there was no significant coronary sinus obstruction. Similar precautions may well be desirable in further applications of coronary sinus catheterization.

The authors are greatly indebted to Drs. Seymour S. Kety, Carl F. Schmidt, Richard J. Bing, Harold E. Himwich and Walter Fleischmann for their helpful advice. Drs. Stanley H. Durlacher and Benjamin H. Landing greatly contributed to the interpretation of the pathological findings. The skillful technical assistance of Mr. William P. McShane, Mr. Robert C. Johnson, Miss Alice Willis, Miss Sarah Bederman and Mrs. Pauline Wilson, as well as the statistical analyses contributed by Miss Frieda Faiman, are gratefully acknowledged.

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MEASUREMENT OF CORONARY BLOOD FLOW BY THE NITROUS OXIDE METHOD¹

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Received for publication November 14, 1947

The nitrous oxide method introduced by Kety and Schmidt (1) for the quantitative measurement of cerebral blood flow in man is applicable in principle to any organ from which a representative sample of venous blood can be collected. The heart, by reason of its essentially homogenous structure and venous drainage into the coronary sinus (predominately from the left ventricle), should be a suitable organ. The applicability of the nitrous oxide method to the coronary circulation of the dog was established by a set of experiments begun more than two years ago but hitherto reported only in preliminary form (2, 3). The potential value of this procedure was greatly enhanced by the recent development of a method for catheterizing the coronary sinus (4), which makes possible quantitative estimations of coronary blood flow and cardiac oxygen consumption in the dog under conditions differing from normal only by the presence of light anesthesia, and even in intact man, once the validity and safety of the procedure have been established. The question of safety has been considered in the preceding paper (4). This report presents data bearing on the validation of the nitrous oxide method for measuring blood flow as applied to the coronary circulation of the dog.

The experiments were of two main types: *a*) those in which the indirect nitrous oxide method was calibrated against simultaneous direct measurement of blood flow by means of a bubble flowmeter; and *b*) those in which the nitrous oxide method was used alone but with variations intended to test its validity and to reveal the best procedure to employ.

I. COMPARISON OF BLOOD FLOW DATA OBTAINED SIMULTANEOUSLY BY THE BUBBLE FLOWMETER AND NITROUS OXIDE METHODS

METHOD. The method used to compare with the nitrous oxide technic was that of the bubble flowmeter (3, 8). Dogs, weighing 15 to 23 kgm. were anesthetized with pentobarbital sodium (30 mgm. per kgm. intravenously with 30 mgm. supplements if needed). We attempted to keep anesthesia minimal. Heparinized blood was circulated from a cannulated right carotid artery through the flow-

¹ The expenses of these experiments were defrayed in part by a grant from the Life Insurance Medical Research Fund to the Department of Pharmacology, University of Pennsylvania.

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meter and thence into a cannulated anterior descending branch of the left coronary artery. The chest was closed, the pneumothorax was reduced, and the animal allowed to resume spontaneous respirations. Bubble flowmeter and nitrous oxide measurements were made simultaneously.

The technic of the nitrous oxide method of measuring blood flow. This was accomplished by the method of Kety and Schmidt (1, 9). In the early experiments, a 40 per cent nitrous oxide and 60 per cent oxygen mixture was used, preceded by a 20 minute period of denitrogenation with 100 per cent oxygen. In the more recent experiments, a mixture containing 15 per cent nitrous oxide, 21 per cent oxygen and 64 per cent nitrogen was used without a period of denitrogenation.

A pair of respiratory, low-resistance flutter valves was attached to the tracheal cannula. The inflow was from a four-liter rubber bag supplied from the tank of nitrous oxide mixture. Precautions were taken to avoid leaks and the possibility of rebreathing the gas mixture.

Before the nitrous oxide technic can be applied to a particular tissue, the partition coefficient of nitrous oxide in that tissue should be known. This was accomplished by *in vitro* experiments using dog and human hearts. The method used was the same as that used by Kety, Harmel, Broomell and Rhode in establishing a partition coefficient for nitrous oxide in cerebral tissue (10). In brief, dog or human hearts were homogenized in a Waring Blender, placed in a tonometer and allowed to come to equilibrium with nitrous oxide gas. The tissue was then analyzed for the nitrous oxide content. The results are summarized in table 1.

The source of blood samples 1. *Venous.* In the first eight experiments, coronary venous blood was obtained from the coronary sinus by means of a catheter inserted through the right auricular appendage and into the sinus a maximum of 2 cm. The results of these experiments have been briefly reported elsewhere (3). Because 6 of the 15 measurements of coronary blood flow indicated gross contamination, we abandoned this source of venous blood. As will be seen below (Table 4), no such contamination occurs when the catheter is introduced from inside the venous system and is free to move with the heart. At the time, however, it seemed advisable to obviate all risk of contamination with blood from the right auricle, and for this purpose a silver cannula was tied into the great cardiac vein and the blood was allowed to flow continuously into a small flask. This blood was returned to the animal at frequent intervals via the femoral vein. Only the experiments in which venous blood was obtained in this fashion have been included in the comparison of the bubble flowmeter and the nitrous oxide methods.

2. *Arterial.* Arterial blood samples must be withdrawn from the efferent limb of the bubble flowmeter. If this is not done, the venous concentrations of nitrous oxide develop abnormally slowly in relation to the arterial concentrations. The explanation is that arterial blood to the area being studied must pass through the bubble meter before reaching the coronary circulation, the transit requiring an average of one minute. If the arterial samples are not withdrawn from the efferent limb of the meter, then the sampling time is one minute earlier than the time

the blood enters the myocardium. This factor has led to difficulties in investigations of the cerebral circulation (9, 11).

Care of the blood syringes. Blood samples were withdrawn into oiled syringes, the tips of which were filled with heparin. Arterial, coronary venous and mixed venous samples were collected synchronously. The syringes were then capped, immediately immersed in ice water and placed in a refrigerator until the bloods were analyzed for nitrous oxide content (1). Blood samples for oxygen and carbon dioxide determinations were treated in the same manner and were analyzed by the method of Van Slyke and Neill.

RESULTS. A simultaneous comparison of the nitrous oxide and bubble flow-meter methods of measuring coronary blood flow has been made 17 times in 10 experiments. The average results are shown in table 2. In general, there was a satisfactory agreement between the two methods.

TABLE 1. HEART: BLOOD PARTITION COEFFICIENT OF NITROUS OXIDE

	NO. ANALYSES	AVG. COEFFICIENT	STANDARD ERROR
Dog.....	5	1.05	0.013
Human.....	5	1.13	0.013

TABLE 2. COMPARISON OF MEAN VALUES FOR CORONARY BLOOD FLOW MEASURED SIMULTANEOUSLY BY THE BUBBLE FLOWMETER AND NITROUS OXIDE METHODS
(17 determinations in 10 experiments)

BUBBLE FLOW-METER	NITROUS OXIDE	COEFFICIENT OF CORRELATION	PARTITION COEFFICIENT
cc/100G/min. 63.7	cc/100G/min. 67.8	0.77	0.97 s.e. ± 0.052

An analysis of the data (table 2) indicates that the flows obtained were not identical. Several factors may have contributed to this discrepancy. (a) There may have been an overlap of arterial supply to the area of the heart whose venous drainage was being studied. In order to obtain complete agreement between the two technics, all blood in the venous sample must be supplied through the bubble meter. The importance of the site of collection of the arterial samples mentioned before indicates that arterial blood in the area under measurement reached a given concentration of nitrous oxide one minute (more or less depending on the rate of flow) after arterial blood which did not pass through the meter. (i.e. in intact coronary arteries). If these two arterial sources overlapped, the resultant venous nitrous oxide concentration would rise faster than it should and the nitrous oxide flow would be too rapid.

This overlap unquestionably existed in many instances. In only a few experiments did temporary occlusion of the arterial inflow cause cessation of the venous outflow. Also, we became convinced that the venous drainage of the heart was too widespread to say that any one vein drains only a specific area supplied by a

specific artery. In bubble meter experiments performed with the chest open, we have injected a dye (Evans Blue) into the efferent tube of the meter and have seen it appear in all the veins visible on the heart, right and left ventricular wall alike. Prinzmetal, *et al.* (12) have obtained more precise results on this subject.

(b) Our method of establishing blood flow per hundred grams of left ventricle per minute (8) may have led to errors. The procedure used was to terminally inject a solution of Evans Blue dye into the efferent tube of the meter and sacrifice the animal before recirculation could occur. The dyed portion of the heart was then carefully cut away from the unstained portion and weighed. The metered flow was considered as flow to this area and on this basis flow per hundred grams of left ventricle per minute was established. Overstaining, understaining or poor separation of dyed and undyed areas are all possibilities.

In view of the overlap mentioned, it is surprising that the agreement between the two methods was so close. The results therefore indicated that the nitrous oxide method was capable of yielding valid measurements of coronary blood flow and should receive a trial in the intact animal.

Since the constant of partition as determined from the bubble meter experiments was 0.97 and that from the *in vitro* dog heart experiments was 1.05, we felt that a value of 1.0 could reasonably be assigned to this coefficient.

II. MEASUREMENT OF CORONARY BLOOD FLOW IN THE INTACT DOG BY THE NITROUS OXIDE METHOD ALONE

Since the two methods showed satisfactory correlation when used simultaneously, we were ready to proceed with estimations of coronary blood flow by the nitrous oxide method with the newly developed procedure for catheterizing the coronary sinus of the intact dog (4) as soon as the latter became available. Because of the contamination encountered in the bubble flowmeter experiments (presumably with blood from the right auricle) when a catheter was introduced by direct exposure into the coronary sinus (3), we sought at first to have the catheter tip penetrate as far as possible into the coronary venous system. For this purpose we used large dogs and advanced the tip of the catheter to a point just inside the great cardiac vein, meaning that the tip was at least 3-4 cm. from the ostium of the coronary sinus. As a final precaution we withdrew the coronary blood samples at a uniform rate calculated to be less than that at which blood was entering the coronary veins, i.e. no faster than 6 cc. in 30 seconds.

These precautions were taken in all the following experiments.

METHODS. In ten experiments, the coronary sinus was catheterized by the method previously described (4). A slow flow of normal saline was allowed to pass through the catheter. Mean arterial blood pressure was recorded from a femoral artery by a mercury manometer. In all but three experiments the animals were allowed to recover.

The nitrous oxide was administered through an endotracheal tube. Passage of air around the tube was prevented by an inflated balloon on the tube or by carefully packing the pharynx. The respiratory flutter valve and the nitrous oxide administration were as described before. Blood samples were withdrawn

through manifolds (see (9)) connected to the femoral artery and coronary sinus catheters.

RESULTS. The mean control values of ten experiments in which coronary blood flow was measured 17 times are summarized in table 3. It can be seen that by this method coronary blood flow per hundred grams of left ventricle was slightly above the value previously reported from control values obtained with the bubble flowmeter on another group of experiments. The oxygen consumption figure was correspondingly slightly higher. The lower coronary venous oxygen content in these experiments was probably a reflection of the lower arterial oxygen content. It is of note that the coronary arterio-venous oxygen difference was approximately the same in both groups.

In the course of these experiments we came to believe that the contamination observed in the early experiments probably did not mean inevitable admixture

TABLE 3. COMPARISON OF MEAN CONTROL VALUES OBTAINED SEPARATELY BY THE NITROUS OXIDE AND THE BUBBLE FLOWMETER METHODS

	NITROUS OXIDE		BUBBLE FLOWMETER*	
	Control values	Coeff. var.	Control values	Coeff. var.
No. experiments.....	10		10	
No. observations.....	17		19	
Coronary flow (cc/100G/min.).....	71.3	15.7	66.0	13.0
Oxygen consumption (cc/100G/min.)..	9.5	19.1	8.8	13.0
Art. O ₂ content (vol. per cent).....	16.4	11.5	19.0	13.2
Cor. venous O ₂ cont. (vol. per cent)....	2.9	25.1	5.7	44.0
Mean art. B.P. (mm. hg).....	129	9.1	124	19.0
Cardiac rate.....	156	11.8	175	20.8

* (3)

of blood from the right auricle with the sample collected from the catheter in the coronary sinus. Reasons for this belief are given below (see Discussion). Since insertion of the catheter into the coronary veins was found to increase the incidence and severity of pathological changes in the myocardium (see 4), shallow insertion would be preferable if it could be proved equal to deep insertion from the standpoint of collection of uncontaminated coronary venous blood. Accordingly two final groups of experiments were made in which the catheter tip was a) not more than 2 cm. and b) 4-5 cm. inside the ostium. The results are summarized in table 4. In these experiments the shallow insertion led to significant contamination in only 2 out of the 10 cases as compared with 5 out of 7 of the deeper insertions. The values for coronary flow and cardiac oxygen consumption were significantly higher in the shallow insertion than in the deeper ones. However, a very recent series of 4 cm. insertions has yielded values comparable with those obtained in these animals with 2 cm. insertion, and this difference at least may turn out to have been fortuitous. At present it seems proper to conclude only that the position of the tip of the catheter is immaterial

so long as it is well within the coronary sinus and does not obstruct the flow in any of its tributaries.

DISCUSSION. In analyzing this method of measuring coronary blood flow, several important questions arise.

1. Many of the curves drawn from the data indicate some evidence of 'contamination'. By 'contamination' is meant the failure of the venous curve to approach the arterial as a single exponential function. Two primary factors could be involved here, either working in combination or separately: a) Admixture of coronary venous blood with mixed venous blood and b) the presence of another tissue which has a slower blood flow and/or a greater nitrous oxide capacity than cardiac muscle, viz. fat.

Mixed venous blood could come from either retrograde flow into the coronary sinus or from retrograde flow through Thebesian channels from either the right or left ventricle as suggested by the work of Prinzmetal, *et al.* (12). If retrograde flow occurred from either of these sources, one would expect the degree of contamination to vary widely from animal to animal. This has not been the case. In the experiments summarized in table 3, the degree of 'contamination', as determined by the nitrous oxide curves, amounted to an average of 4.4 per cent with a range of 0 to 14.5 per cent. Even in the experiments summarized in table 2 where the venous blood was obtained only from the great cardiac vein, thus ruling out retrograde flow from all sources but Thebesian channels from the left ventricle, there was an average of 3.0 per cent 'contamination'.

In an endeavor to obtain data of a more unequivocal nature, we have attempted several experiments in which samples from the coronary sinus catheter were withdrawn during the injection of an 0.1 per cent solution of Evans Blue Dye into the inferior vena cava or into the right auricle. In none of these experiments was there any evidence of gross contamination of the coronary venous blood, but the method was not delicate enough to give evidence as to the source of the 4.5 per cent 'contamination' noted with the nitrous oxide flows. We believe that the absence of variability from animal to animal, the evidence obtained by the dye injection, and the infrequency of contaminated curves from shallow insertion of the catheter is strong enough to indicate that the 'contamination' is not from the admixture of coronary venous blood with other venous blood.

If one assumes therefore, that it is tissue other than cardiac muscle, probably fat, that causes the 'contamination', then the blood flow values as reported herein are probably somewhat less than true coronary flow. It may be possible, by what promises to be a fairly complex mathematical analysis (13), to resolve the compound curves into their pure components. Until such time, however, the relative insignificance of the factor of 'contamination' permits our values to represent a satisfactory approximation of true myocardial blood flow.

2. A second question of major importance is, does the myocardium come into equilibrium with the arterial nitrous oxide content by the end of ten minutes? We were unable to devise a method of administering nitrous oxide to a dog and then obtaining anerobic samples of the myocardium for analysis. In investigating this factor as applied to the brain, Kety *et al.* (10) found that the brain did

come into equilibrium with the cerebral venous blood within ten minutes. On comparing the capillary density of the myocardium and the brain (see Craigie, 14, for the brain and Wearn, 15, for the myocardium) it was found that the myocardium is approximately five times more vascular than the brain; thus the heart should reach equilibrium even more rapidly than the brain.

3. When one applies this method to the measurement of coronary blood flow, is the result a measure of coronary blood flow for the entire heart or does it apply to a specific area? Venous blood appearing in the coronary sinus is primarily an outflow from the left ventricle (16, 17). However, apparently considerable amounts of blood from other sources may appear in the sinus (12). While we have made no measurements of blood flow in the right coronary artery nor of the oxygen consumption of the right ventricle, there seems to be no reason why these two factors should behave differently than they do in the left ventricle. However, due to the work differential, rate of flow and oxygen utilization by the right ventricle may differ from that which is occurring simultaneously in the left

TABLE 4. COMPARISON OF MEAN VALUES OBTAINED WHEN CORONARY VENOUS BLOOD WAS COLLECTED 2 CM AND 4 OR MORE CM INSIDE THE OSTIUM

	4+ cm		2 cm	
	Control values	Coeff. var.	Control values	Coeff. var.
No experiments.....	6		7	
No observations.....	7		10	
Coronary flow cc/100G/min.).....	74	7.5	86	11.0
Oxygen consumption (cc/100G/min.).....	9.1	8.0	12.3	17.0
Art. O ₂ content (vol. per cent).....	16.8	14.0	18.0	7.0
Cor. venous O ₂ content (vol. per cent).....	4.2	25.0	3.6	26.0
Mean art. B.P. (mm Hg.).....	126	13.0	134	11.0
Cardiac rate.....	134	16.0	145	8.0

ventricle. This was well shown by the work of Moe and Visscher (18). The admixture of the blood in the coronary sinus from right and left ventricle is probably not sufficient for the sinus blood to represent a true average of all coronary venous blood. Thus we prefer to consider the figures obtained from the nitrous oxide flows as representing left ventricular blood flow.

Difficulties and limitations of the method as applied to the heart. 1. Occasionally after the catheter has been introduced into the coronary sinus, it is not possible to get a free withdrawal of blood. The apparent explanation for this difficulty lies in the structure of the coronary sinus and the veins opening into it. At the opening of each major vein into the coronary sinus there are valves of the semilunar type (19). The valve that guards the opening of the great cardiac vein into the coronary sinus lies at the end of the sinus and thus directly in the path of the catheter should it be introduced 3 cm. or more. If the catheter tip gets into the pocket of this valve, it cannot be readily advanced nor can blood be withdrawn from it since the valve flap effectively occludes the tip. Careful

manipulation of the catheter may remove it from this pocket, or if the tip of the catheter has multiple openings, blood may be withdrawn.

The sharp curvature of the sinus may interfere to some extent if the catheter is not sufficiently flexible. Here, the tip of the catheter impinges against the wall of the sinus and withdrawal of blood becomes difficult. Again, a catheter with multiple openings at the tip is useful.

2. If the catheter is advanced too far into the great cardiac vein or if the catheter is too large, a complete or partial obstruction of the venous return into the coronary sinus might occur. A marked pulsation of the saline in the Murphy Drip attachment to the catheter suggests this complication. Such an obstruction would not only make nitrous oxide determinations unreliable but would also invalidate the oxygen and carbon dioxide findings. Using large dogs, medium-size catheters (size 7 or 8F) and by advancing the tip of the catheter about 2-4 cm. into the sinus as determined by fluoroscopic vision, we could usually obviate this difficulty.

3. Trauma to the coronary sinus system has been noted in our series. This has been discussed in the preceding paper (4) and needs no further comment here.

4. The nitrous oxide method of measuring blood flow can be applied only if the conditions under which the flow is measured remain static for at least the ten minutes required for measuring the flow. This decreases the value of the method for the study of the action of many comparatively shorter-acting drugs.

Subject to these limitations, we believe that the nitrous oxide method of measuring coronary blood flow is practicable. The method has yielded results comparable to those obtained by the bubble meter in the dog. In view of the untoward pathological findings in the dog, the adaptation of this method to man must be made with caution. However, one cannot overlook the fact that the coronary sinus of man has been catheterized many times incidental to right auricular catheterization (5-7), and there has been as yet no indication that the procedure is dangerous.

SUMMARY

The nitrous oxide method of measuring blood flow has been investigated to determine its adaptability to the coronary circulation. The method has been calibrated by simultaneous bubble flowmeter determinations. Control values for measurements of the coronary blood flow of the dog by this method are presented and compared with similar control values obtained previously with the bubble flowmeter. The difficulties and limitations of the method in its application to the coronary blood flow are discussed. Untoward effects have become less frequent and severe as our experience and skill have increased but they have not been eliminated by any refinements thus far developed.

The authors wish to express their appreciation to Dr. Carl F. Schmidt for his valuable suggestions and criticisms offered during this investigation. They also wish to acknowledge the assistance of Dr. E. L. Foltz in some of the experiments and the technical help given by Miss H. T. Broomell, Mrs. C. B. Rhode, M. Paul, and W. Tuddenham.

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NORMAL HUMAN ARTERIAL OXYGEN SATURATION DETERMINED BY EQUILIBRATION WITH 100 PER CENT O_2 IN VIVO AND BY THE OXIMETER¹

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Received for publication November 13, 1947

Until recently, the normal range for the saturation of arterial hemoglobin with oxygen has been considered to be 93 to 97 per cent. In 1944, Roughton, Darling and Root (1) analyzed critically certain errors that may occur in the determination of the percentage saturation of hemoglobin with oxygen and concluded that the average normal figure of 95 per cent should be revised upward to 97 per cent. At the same time, Drabkin and Schmidt (2) measured the arterial oxygen saturation in five normal males by a spectrophotometric technique and found an average figure of 98.6 per cent with a very narrow range (98.0 to 99.3 per cent).

Because of the great importance of the estimation of arterial oxygen saturation as an overall test of lung function, we decided to perform further studies upon a larger group to determine *a*) what constitutes normal arterial oxygen saturation and *b*) whether presumably normal individuals vary widely about an average figure or whether normality is confined to very narrow limits.

Roughton, Darling and Root (1) concluded that the errors in the Van Slyke technique lay not in the determination of O_2 content of the arterial blood, but in the estimation of the O_2 capacity by the in vitro equilibration technique. We decided, therefore, to avoid the errors inherent in the in vitro equilibration technique and to determine O_2 capacity by an in vivo equilibration technique employing known concentrations of O_2 in the alveoli. After determining the content of O_2 in the arterial blood while the subject was breathing room air, the capacity of hemoglobin to take up O_2 was found by measuring the arterial O_2 content during inhalation of 100 per cent O_2 . Appropriate corrections were made in each case for the amounts of O_2 present in the bloods in physical solution. An example follows: If the O_2 content of arterial blood taken from a subject during inhalation of room air was 19.3 vols. per cent and the O_2 content of arterial blood taken during inhalation of 100 per cent O_2 was 22.04 vols. per cent, when appropriate subtractions are made to eliminate the O_2 dissolved in physical solution in the bloods at the respective O_2 tensions (3), these figures become 19.0 and 20.0 vols. per cent. The saturation of arterial blood (during inhalation of room air) thus becomes 19/20 or 95 per cent.

¹ The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Pennsylvania. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

This method of 'in vivo equilibration' with 100 per cent O_2 to determine O_2 capacity of arterial blood was carried out in a series of 20 normal males, and the oxygen saturations calculated as above. In addition, for purposes of comparison, arterial oxygen saturations were determined in the same subjects by *a*) the usual technique of determining capacity by in vitro equilibration of blood with room air and *b*) noting the rise in oximeter readings of arterial oxygen saturation upon breathing 100 per cent O_2 .

METHODS

A. General. Twenty normal males between the ages of 18 and 63 served as subjects; 12 were normal medical students and the remainder were ambulatory patients with no known pulmonary disease. Each subject lay quietly in bed for 15–20 minutes. During this time an oximeter was placed upon his ear and sufficient time allowed for full vasodilatation of the ear vessels. The oximeter was set to read 95 per cent saturation. The subject breathed through valves, and the minute volume of respiration was recorded by conducting the expired air into a balanced compensated spirometer equipped for graphic recording. The region around a brachial or femoral artery was thoroughly infiltrated with 1 per cent procaine. A 10 cc. syringe with a #20 gauge intraspinal needle attached was prepared by drawing a small amount of heparin solution into the syringe, pulling the plunger back to the 10 cc. mark so that the walls of the syringe were wet with heparin. The air and the remainder of the heparin were then slowly ejected through the needle. In this way the dead space in the needle and syringe (average 0.15 cc.) was filled with anticoagulant. The needle was then inserted into the artery; in no instance did the subject experience discomfort or know the precise time of arterial puncture. None of the respiratory records showed hypo- or hyperventilation during the withdrawal of blood. Ten cc. were withdrawn, the syringe was disconnected from the needle and the latter was styletted and left in place. The syringe was immediately capped, taken to a Van Slyke manometric gas analyzer prepared to receive it and the analysis for CO_2 and O_2 content was begun at once (blood #1).

As soon as the first blood sample was withdrawn the subject was given 100 per cent O_2 to breathe for 10–15 minutes. This period was considered sufficiently long to produce maximal arterial oxygen levels because *a*) inhalation of 100 per cent O_2 reduces the alveolar air (and presumably the arterial blood) nitrogen to negligible values in five minutes and *b*) oximeter records demonstrate that the arterial oxygen saturation reaches its peak within 2–3 minutes after inhalation of 100 per cent O_2 . After 10–15 minutes, a second sample of arterial blood was withdrawn and immediately analyzed for CO_2 and O_2 content (blood #2).

B. Van Slyke technique. The same 1.0 cc. pipette was used for duplicate analyses. In order to prevent any loss of blood gases during the filling and emptying of the pipette, a modified mercury-filled pipette was used in *experiments 11 to 20* so that the blood was never exposed to air. Before each analysis the blood was mixed thoroughly by agitating with a small amount of chemically clean mercury that had been drawn into the syringe. Two or three analyses

were performed on each sample, the blood being stored in the capped, iced syringes between analyses. The standard error in the series of duplicate determinations was 0.07 per cent; the standard error for the calculated O₂ saturation was 0.5 per cent saturation.

Each sample was also analyzed in the Evelyn photo-electric colorimeter for total hemoglobin pigment after conversion to cyanmethemoglobin. This was done to detect any change in red blood cell-plasma ratio that might have occurred in the circulating blood between the withdrawal of *samples 1* and *2*. In 9 of the 20 subjects, the readings were identical in the two samples; in the others slight corrections were necessary.

Calculations were performed as follows. *Blood # 1*: The oxygen contents obtained in the duplicate analyses were averaged, and 0.3 cc. subtracted from this figure; 0.3 cc. represents the amount of O₂ physically dissolved in arterial blood at 38° C. (3) at the pO₂ of 100 mm. Hg believed to represent average arterial oxygen in normal subjects breathing room air (4, 5). The figure thus obtained represents the amount of O₂ combined with hemoglobin in blood # 1. *Blood # 2*: Duplicate analyses were averaged. If the total pigment, as determined by the determination of cyanmethemoglobin, differed in bloods # 1 and # 2, the O₂ content of blood # 2 was corrected to that both samples represented the same amount of total pigment. Then 2.04 cc. were subtracted; 2.04 cc. represent the amount of physically dissolved O₂ in arterial blood at 38°C. and a pO₂ of 673 mm. Hg. (That the figure of 673 mm. Hg may be too high will be commented upon later.) The resulting figure represents the amount of O₂ combined with hemoglobin in blood # 2. The fully corrected figure for blood # 1 divided by that for blood # 2 represents the arterial O₂ saturation of blood # 1 (that obtained during breathing room air).

This method was checked by an in vitro equilibration technique which was performed as follows: A portion of blood # 1 was rotated in a 50 cc. flask for five minutes at room temperature. Then two pipettes were filled simultaneously in order to obtain blood of the same red blood cell-plasma ratio. One of these was used for analysis for O₂ capacity in the Van Slyke apparatus (blood # 3) and the other for determination of total pigment after conversion to cyanmethemoglobin. The value obtained for O₂ capacity was corrected in two ways: a) 0.56 to 0.63 cc. was subtracted to correct for the amount of physically dissolved O₂ in blood at 154 mm. pO₂ and at temperatures ranging from 29° to 24° C.; b) if the total pigment differed in bloods # 1 and # 3, corrections were made to equalize the total pigments. In 12 of 17 cases the total pigment was greater in the equilibrated sample, thus supporting Roughton's statement that drainage errors in the glass vessel cause the sample to be unduly rich in red blood cells (1).

O₂ saturation was then calculated as:
$$\frac{\text{corrected blood content}}{\text{corrected blood capacity}} \times 100 = \text{per cent O}_2 \text{ saturation.}$$

C. Oximeter technique. After an ear 'warm-up' period of 15 minutes and an instrumental 'warm-up' period of 45 minutes, the oximeter (6) was set to 95 per cent saturation when the subject was breathing room air. The maximal increase

that followed inhalation of 100 per cent O_2 was noted (within two to five minutes after blood #1 was withdrawn). The O_2 saturation of the subject during breathing of room air was considered to be 100 per cent minus the per cent saturation increase that occurred during O_2 inhalation. A similar procedure was carried out in 39 additional subjects on whom oximetry alone was done, and no arterial

TABLE 1. CORRECTED O_2 CONTENTS OF ARTERIAL BLOODS TAKEN DURING INHALATION OF ROOM AIR AND 100% O_2

SUBJECT #	CORRECTED CONTENT		PER CENT SATURATION	O_2 ADDED TO HEMOGLOBIN	SUBJECT #	CORRECTED CONTENT		PER CENT SATURATION	O_2 ADDED TO HEMOGLOBIN
	room air	100% O_2				room air	100% O_2		
				cc.					cc.
1	18.45	19.71	93.6	1.26	11	17.50	17.86	98.0	0.36
2	16.40	16.66	98.4	0.26	12	21.25	22.51	94.4	1.26
3	19.80	20.19	98.1	0.39	13	17.45	18.16	96.1	0.71
4	19.25	20.13	95.6	0.88	14	17.00	17.46	97.4	0.46
5	19.75	19.66	100.5	-0.09	15	18.87	19.13	98.6	0.26
6	18.75	18.99	98.7	0.24	16	16.70	17.28	96.6	0.58
7	16.90	17.06	99.1	0.16	17	15.32	15.82	96.8	0.50
8	21.25	21.99	96.6	0.74	18	17.50	17.56	99.7	0.06
9	20.40	20.54	99.3	0.14	19	18.63	18.43	101.0	-0.20
10	18.05	18.66	96.7	0.61	20	18.15	19.09	95.1	0.94

¹ Corrected a) by subtraction of amount of physically dissolved O_2 ; b) by equilization of total hemoglobin pigments in the two samples.

TABLE 2. COMPARISON OF O_2 SATURATIONS ESTIMATED BY THREE METHODS

SUBJECT #	IN VIVO TECH.	IN VITRO TECH.	OXIMETER TECH.	SUBJECT #	IN VIVO TECH.	IN VITRO TECH.	OXIMETER TECH.
1	93.6	—	94.5	11	98.0	94.7	96.0
2	98.4	—	98.0	12	94.4	96.4	95.0
3	98.1	98.4	97.5	13	96.1	95.2	97.0
4	95.6	97.5	96.5	14	97.4	—	94.0
5	100.5	98.8	95.0	15	98.6	95.7	96.0
6	98.7	99.2	97.0	16	96.6	98.5	95.5
7	99.1	97.0	97.0	17	96.8	95.0	97.0
8	96.6	98.8	95.0	18	99.7	101.4	98.0
9	99.3	97.2	96.5	19	101.0	100.0	95.0
10	96.7	98.1	97.0	20	95.1	93.2	—
Average.....					97.5	97.4	96.2

samples were withdrawn. In all 58 cases, ear thickness readings were noted frequently to be certain that conditions were steady throughout the experimental period.

RESULTS

In table 1 are shown the corrected O_2 contents of 20 normal basal individuals breathing room air and 100 per cent O_2 . On the basis of these figures, the O_2

saturation of the arterial blood averaged 97.5 per cent, with a range of 93.6 to 101.0 per cent. Eight of 19 determinations varied from the average by more than 2 S.E. of the method.

In table 2 is shown a comparison of the O_2 saturations determined in these subjects by the three methods. The average figures for the 'in vivo' and 'in vitro' saturations show excellent agreement; the individual differences may be accounted for largely by a summation of experimental errors in the determination of oxygen content and total pigment. The average saturation determined by the oximeter technique was only 96.2 per cent.² The standard error of the mean of the differences between the 'in vivo' equilibration and the oximeter in the 19 cases was 0.5 and the t value was 3.0, so that the difference is clearly significant.

DISCUSSION

Several possible sources of error in our methods should be considered. First of all, the absolute values for O_2 saturation depend upon the estimation of dissolved O_2 in the two blood samples. The amount of dissolved O_2 depends upon the arterial pO_2 and the αO_2 at 38°C. The arterial pO_2 was not measured but estimated. The arterial pO_2 of normal males breathing room air at rest is known to be about 100 mm. Hg (4, 5), and a small variation here would not alter the results appreciably. On the other hand the arterial pO_2 during inhalation of '100 per cent' O_2 may be less than 673 mm. Hg for several reasons: *a*) the O_2 used contains only 99.7 per cent O_2 ; *b*) small amounts of N_2 must enter the alveolar air from the venous blood during the experimental period; and *c*) the arterial pO_2 may be 10 to 30 mm. below the alveolar pO_2 for a variety of reasons (7, 8). If the arterial pO_2 were as much as 30 mm. below the alveolar pO_2 , this would tend to reduce the arterial saturations (calculated by the in vivo equilibration technique) by approximately 0.5 per cent.

We have used in this study the average figure for αO_2 at 38°C. determined for whole ox blood by Sendroy, Dillon and Van Slyke (3). It is possible that this varies slightly from species to species and from individual to individual. No allowances have been made for this.

While the in vivo equilibration technique is useful experimentally, it is not applicable to clinical usage, for it can be employed only when the arterial pO_2 is measured or can with reasonable accuracy be estimated. In patients with cardio-respiratory disease, large errors may occur if arterial pO_2 is assumed during inhalation of either room air or of 100 per cent O_2 .

It appears from this study, as from a previous investigation (1), that the in vitro equilibration technique for O_2 saturation can be made more accurate by correcting for drainage errors and for dissolved O_2 (3). Many clinical laboratories measuring O_2 saturation by the in vitro technique fail to make either correction. Failure to make the simple correction for dissolved O_2 results in a figure for O_2 saturation of 1.5 per cent below the true value, if the equilibration was carried out at room temperature.

² Dr. W. M. Boothby of the Mayo Foundation determined arterial oxygen saturation by the oximeter technique in a large group of normal men and found it to be approximately 97 per cent (personal communication).

It should also be pointed out here that the error is such in the determination of O₂ saturation by manometric techniques that significance should not be attached to small differences.

The error appears to be greater in the use of the oximeter. First of all, the average saturation was significantly lower by this technique. In addition, oximeter increases of 5 per cent occurred in *subjects 5 and 19*, both of whom were completely saturated by one or both of the other techniques. While this may be explained as a summation of errors, it is possible that the oximeter scale may be incorrect in the 95–100 range (expanded too much) or that the oximeter may be analyzing blood that contains slight admixtures of venous blood (either because of incomplete capillary dilatation, because of O₂ consumption by the ear tissues, or because of vasoconstriction induced by oxygen inhalation).

We have employed the oximeter increase on breathing O₂ as a rough measure of the original arterial O₂ saturation. The limitations of this are obvious: the oximeter measures *changes* and not *absolute* saturations, unless the oximeter is first set at a known value. In our experience with 58 normal subjects, inhalation of O₂ raised the oximeter reading an average of 3.8 with a S.D. of 1.36 and a range of 2 to 6 per cent. Thus an increase upon breathing O₂ of more than 8 per cent (average + 3 S.D.) indicates definite anoxemia. The reverse is not true, i.e. a change of less than 8 per cent does not necessarily rule out anoxemia. In our experience, however, the *rate* of increase of arterial O₂ saturation is a more sensitive indicator of cardiorespiratory abnormality than is the *amount* of increase (9).

The results obtained in this study by the *in vivo* and *in vitro* techniques agree with the findings of Roughton *et al.* (1) that average normal arterial O₂ saturation is greater than 95 per cent and closer to 97.5 per cent. The latter figure is 1.0 to 1.5 per cent lower than that obtained by Drabkin and Schmidt (2). The variability in our subjects cannot be explained wholly on the basis of the experimental errors listed. It is possible *a*) that a lower average arterial O₂ saturation occurs in some individuals because of improper mixing or diffusion of gases in or through circulated alveoli or because of other shunts or *b*) that some methemoglobin or carbon monoxide hemoglobin exists in the arterial blood of certain individuals; these may be converted into O₂ carrying hemoglobin in the presence of a high arterial pO₂. Methemoglobin concentration was not determined in this study. Van Slyke *et al.* (10) found an average of only 0.4 per cent methemoglobin in freshly drawn venous blood and noted that in about half the bloods its concentration was so low as to be uncertain. The mean total 'inactive' hemoglobin was found to be 1.3 per cent in their subjects. Van Slyke did not determine these values upon arterial blood; it is possible that 'inactive' hemoglobin would be less or absent in arterial blood, as indicated by the work of Drabkin and Schmidt (2).

The blood of *subject 20* was tested for carbon monoxide hemoglobin by a very sensitive method by Dr. Spealman and none could be found. Since the blood of this subject was 95.1 per cent saturated, this low figure could not have been accounted for by the presence of carbon monoxide hemoglobin in blood #1 and its absence in blood #2 of the *in vivo* equilibration technique. No other tests

for COHb were made, but all of the smoker-subjects (with one exception) had not smoked for several hours to several days before the tests.

SUMMARY

The arterial oxygen saturation was determined in 20 subjects by an *in vivo* equilibration technique which avoids the chief errors encountered by the usual *in vitro* technique.

Normal arterial oxygen saturation by this technique averaged 97.5 per cent.

Saturations were measured in the same subjects by a corrected *in vitro* equilibration technique and by the oximeter. The former yielded an average figure of 97.4 per cent and the latter, 96.2 per cent. The usefulness of and errors in these techniques are discussed.

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STUDIES OF THE PULMONARY CIRCULATION AT REST AND DURING EXERCISE IN NORMAL INDIVIDUALS AND IN PATIENTS WITH CHRONIC PULMONARY DISEASE^{1, 2}

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Received for publication December 22, 1947

Studies of the cardiac output of normal individuals during muscular exercise have been made by several investigators (1-5). Systemic blood pressure has likewise been measured during exercise, making possible the calculation of peripheral resistance and the work of the left ventricle. Until recently (5), such information was not available in the case of the right ventricle because pulmonary arterial pressure was not known. By using the technic of pulmonary artery catheterization, both cardiac output and pulmonary arterial pressure can be determined during exercise, and pulmonary resistance and the work of the right ventricle can be calculated. This report deals with such studies on three normal individuals and eight patients with chronic pulmonary disease from whom data were obtained at rest and during exercise.

In some patients with chronic pulmonary disease the pulmonary arterial pressure and cardiac output at rest are normal (6, 7), and it is of interest to determine whether the pulmonary arterial pressure becomes abnormally high during exercise. In these patients and in others who show high pulmonary arterial pressure at rest it is of importance from the point of view of estimating their disability to determine whether there is abnormally low cardiac output or abnormally high work of the right ventricle during exercise.

METHODS

In most instances the subjects were given mild sedation on the night before and the morning of the studies and came to the laboratory without breakfast. The normal subjects took no sedation and had orange juice, black coffee, and dry toast for breakfast at least an hour and a half before the first samples were taken.

The catheter was introduced through the basilic vein or one of its tributaries with the subject lying on a horizontal fluoroscope, and the tip of the catheter was placed in the truncus of the pulmonary artery (8-11). An indwelling arterial needle was placed in the brachial artery on the other side. Resting pulmonary

¹ Under grants from the Commonwealth Fund and the Life Insurance Medical Research Fund.

² W. F. Hamilton and J. W. Remington, Department of Physiology, University of Georgia School of Medicine, collaborated on a comparison of cardiac output methods in which some of the data herein presented were used.

and brachial artery pressures were recorded with a Hamilton manometer and samples were taken for cardiac output determination. The subject then stood up, walked across the room, and seated himself on a bicycle ergometer. During the transfer the saline infusion through the catheter was continued by holding the flask high, and the arm with the arterial needle was kept extended. In spite of slight motion of the arms and head during exercise, no great difficulty was encountered in withdrawing blood samples, collecting expired air or recording pulse-pressure curves.

All exercise measurements were taken in a 'semi-steady' state, i. e., after six or seven minutes of exercise at a constant rate and after the pulmonary ventilation had assumed an approximately constant value. The work load was varied by applying a brake and by changing the rate of pedaling, but was calibrated only on the basis of the subject's oxygen consumption in ml. per minute. Pulmonary and brachial artery pulse-pressure tracings during exercise were taken with an electrical type of manometer (12). The zero point for pulmonary pressure measurements was taken as the third intercostal space anteriorly. Pulmonary arterial pressure recordings taken with the subject seated on the bicycle before exercise served as controls and were comparable to pressures measured in the supine position. The tracings showed more artefacts during exercise than at rest because of motion of the catheter, and therefore the values for systolic and diastolic pressure were somewhat less accurate than the corresponding values at rest. Mean pressures, calculated by planimetric integration of the pulse-pressure curves throughout several complete respiratory cycles, were probably not in error by more than 2 mm. Hg.

Vascular resistances in the pulmonary circuit and in the greater circuit were calculated according to the formulae:

$$R \text{ (pulmonary)} = \frac{P. A._m \times 1.332 \times 60}{C. O.}$$

$$R \text{ (peripheral)} = \frac{B. A._m \times 1.332 \times 60}{C. O.}$$

where R = vascular resistance in dynes-sec.-cm.⁻⁵

P. A._m = mean pressure in the pulmonary artery in mm. Hg

B. A._m = mean pressure in the brachial artery in mm. Hg

C. O. = cardiac output in l. per minute.

Since neither the left nor the right atrial pressure was known, neither was introduced into the expression for pressure drop in the numerator of the equations. Atrial pressure is close to zero in the absence of heart failure or valvular disease, however, and any error resulting from the omission of this factor was not great in the cases under consideration. There is some evidence that the mean pressure in the left auricle is a few mm. Hg higher than in the right (13), and therefore resistance in the pulmonary vascular bed as calculated may tend to be over-estimated.

The conventional formulae were used to make a rough approximation of the work of the ventricles against pressure:

$$W \text{ (right ventricle)} = C. O. \times P. A._m \times 0.1332$$

$$W \text{ (left ventricle)} = C. O. \times B. A._m \times 0.1332$$

The work of the ventricles is more accurately determined by using arterial pressures during systole rather than over the entire cardiac cycle. When this is done, the results come out 10 to 40 per cent higher, according to a recent publication by Remington and Hamilton (14), because the mean pressure during systole is 10 to 40 per cent greater than the mean pressure over the whole of the cardiac cycle. We have not calculated ventricular work in the approved manner because of the extreme irregularity of the pulse-pressure tracings during exercise which would have necessitated an arbitrary decision as to the duration of systole. Since we will be concerned with relative rather than absolute values for the work of the right and left ventricles, the conventional formulae are believed to provide an adequate estimate of work done against pressure.

In making physiological interpretations we should like to know how closely work done against pressure approximates the total work of the ventricles. It is therefore necessary to know the order of magnitude of kinetic work. Authorities agree that it is insignificant at rest, but since it varies as the cube of the volume flow of blood and inversely as the fourth power of the radius of the vessel into which the blood is ejected (15), any change in these factors occurring during exercise must be reflected many times over in the value for kinetic work. Accurate information regarding the radii of the pulmonary artery and aorta during work is not available, and the effect of various chronic pulmonary diseases upon the distensibility of these vessels is difficult to estimate during life. Sample calculations for normal subjects suggest that kinetic work constitutes approximately 3 per cent of the total work of the right ventricle at rest and that the percentage increases considerably during heavy exercise (15, 16). The kinetic work of the left ventricle is approximately the same as that of the right in absolute terms but constitutes a much smaller percentage of total left ventricular work. Disease states which narrow the pulmonary artery or reduce its distensibility must increase the kinetic work of the right ventricle.

MATERIAL FOR STUDY

The three normal subjects were physicians in good health, and the eight patients suffered from various types of chronic pulmonary disease. Their physical characteristics and diagnoses are shown in table 1.

RESULTS

Normal subjects. In table 2 the data obtained on the normal subjects are presented and the average values for rest and two grades of exercise are calculated. For the milder exercise the oxygen consumption varied from 269 to 512 ml. per minute per square meter of body surface; for the more severe work the range was

TABLE 1

CASE NO.	DIAGNOSIS	AGE	SEX	HT.	WT.	B.S.A.
				cm.	kgm.	sq. m.
377	Normal	36	M	176	62.8	1.78
378	Normal	32	M	178	66.6	1.84
379	Normal	26	M	178	70.5	1.87
366	Left pneumonectomy, partial thoracoplasty, for tuberculous bronchial stenosis	34	F	150	48.0	1.41
371	Chronic pulmonary tuberculosis, left fibro-thorax, re-expanded pneumothorax	34	F	160	65.8	1.71
372	Nodular pulmonary fibrosis, cause undetermined; chronic pulmonary emphysema, moderate	39	F	160	48.5	1.48
383	Silico-tuberculosis, moderate extent	39	M	181	84.0	2.05
354	Post-traumatic left fibrothorax, mediastinal dislocation	44	F	160	66.0	1.69
368	Chronic pulmonary emphysema, pulmonary fibrosis healed tuberculosis	40	M	165	59.0	1.65
358	Saccular bronchiectasis, recent acute broncho-pneumonia with mild decompensation	54	M	178	74.5	1.92
344	Thrombosis of the left pulmonary artery, mild cardiac decompensation	41	F	154	47.0	1.42

TABLE 2

NO.	STATE	HEART RATE	O ₂ Hb SATURATION (%)	O ₂ Hb CONSUMPTION (ml./min./M ² .S.)	A-V DIFFERENCE (VOL.%)	CARDIAC OUTPUT (L./MIN./M ²)	STROKE VOLUME (ml./BEAT)	BLOOD PRESSURES, (MM. HG)						VASCULAR RESISTANCE DYNES SEC. CM ⁻⁵		WORK OF THE VENTRICLES AGAINST PRESSURE		
								Brachial artery			Pulmonary artery			Peripheral	Pulmonary	Left joules/min.	Right	
								syst.	diast.	mean	syst.	diast.	mean				joules/min.	joules/min./m ²
377	Rest	47	97	111	4.2	2.62	99	120	59	82	20	10	15	1400	249	51	9	5
	Exercise (1)	77	95	436	7.6	5.68	131	144	83	112	24	3	8	900	60	150	10	6
	Exercise (2)	105	94	700	9.2	7.53	128	152	100	130	19	0	10	750	57	230	17	10
378	Rest	91	99	147	3.8	3.83	64	122	73	94	19	8	12	1060	135	89	11	6
	Sitting	100	—	—	—	—	—	—	—	—	18	9	13	—	—	—	—	—
	Exercise (1)	127	97	512	8.2	6.17	90	133	88	108	21	5	12	835	93	160	18	10
	Exercise (2)	151	96	912	9.9	9.11	111	168	101	131	20	6	8	625	39	292	18	10
379	Rest	73	96	120	3.6	3.35	86	93	63	76	—	—	11	972	144	63	9	5
	Exercise (1) ¹	90	99	269	6.2	4.36	91	97	64	79	—	—	13	752	123	86	14	8
	Sitting	86	—	—	—	—	—	—	—	—	—	—	13	—	—	—	—	—
	Exercise (2)	157	97	824	11.6	7.14	85	140	83	107	—	—	14	634	84	190	25	13
Average of 3 normals																		
Rest		70	97	126	3.9	3.27	83	112	65	84	20	9	13	1144	176	68	10	5
Exercise (1)		98	97	406	7.3	5.40	104	125	78	100	23	4	11	829	92	132	14	7
Exercise (2)		138	96	812	10.2	7.93	108	153	91	123	19	3	10	680	60	237	20	11

¹ Leg exercise performed in the supine position.

from 700 to 912. Because of the differences in oxygen consumption per square meter, the figures for arterio-venous difference and cardiac output cannot be compared directly. In two of the three normal subjects the stroke volume increased significantly with exercise; in the third there was little change in stroke volume. There was a fall in pulmonary arterial mean pressure with increasing work in two of the three normal subjects. The third subject showed an insignificant rise. The fall in mean pressure was due to a fall in diastolic pressure; the systolic pressure remained unchanged. When one considers that the cardiac output increased on the average two and one half times, it is obvious that a de-

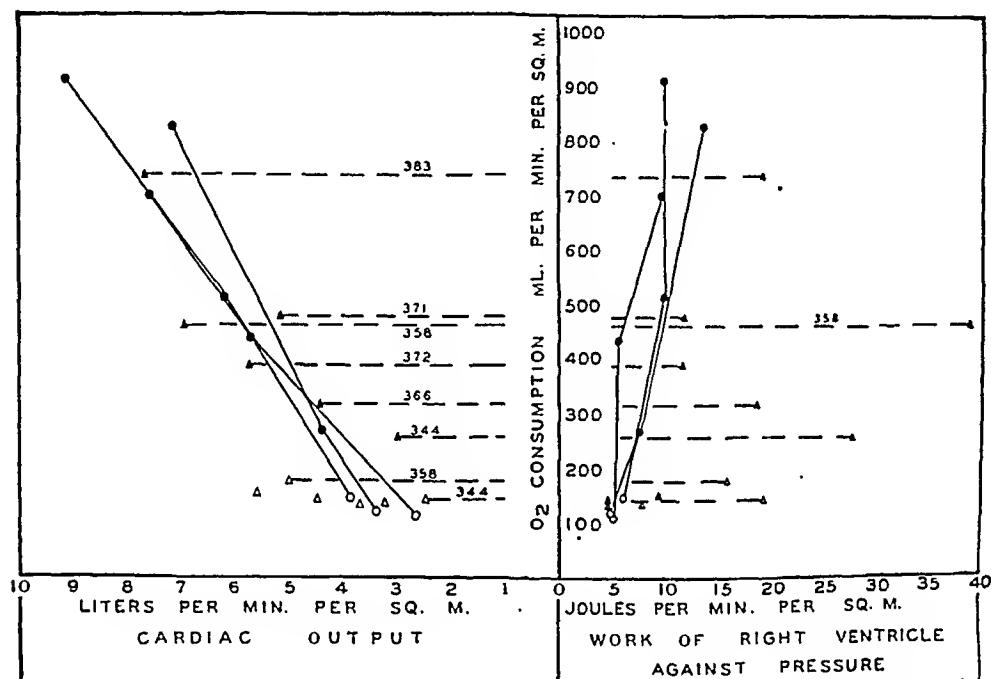


FIG. 1. RELATIONSHIPS BETWEEN OXYGEN CONSUMPTION, cardiac output, and work of the right ventricle against pressure. *Circles* represent normal subjects; *triangles* represent patients with chronic pulmonary disease; *open circles* and *triangles* represent resting values; *closed circles* and *triangles* represent values obtained during exercise; *solid lines* connect points obtained on the same normal subject at different metabolic levels; *broken lines* connect points obtained on patients during a single study.

crease in mean pressure could occur only in association with a sharp fall in pulmonary vascular resistance. As seen in the table, pulmonary vascular resistance decreased to approximately one third of its resting value during the more severe exercise. The estimated work of the right ventricle against pressure increased on the average only 10 joules per minute during exercise while the work of the left ventricle increased 170 joules per minute.

In figure 1 the cardiac output at different levels of oxygen consumption can be studied in relation to the work of the right ventricle against pressure. The solid lines connect points obtained on the normal subjects. The almost vertical slope of the curves on the right indicates that the increase in right ventricular

work against pressure was very small. There was marked similarity in the responses of the three normal individuals both with respect to cardiac output and with respect to the work of the right ventricle.

Patients with chronic pulmonary disease. The patients who were studied had a wide range of pulmonary pathology including fibrothorax, bronchiectasis, pneumonectomy, fibrosis, emphysema, silico-tuberculosis, and thrombosis of the left

TABLE 3

NO.	STATE	HEART RATE	O ₂ HD SATURATION (%)	OXYGEN CONSUMPTION (ML/MIN/M ² B.S.)	A-V DIFFERENCE (VOL %)	CARDIAC OUTPUT (L/MIN/M ²)	STROKE VOLUME (ML/BEAT)	BLOOD PRESSURES (MM. HG)						VASCULAR RESISTANCE DYNES/SEC. CM ⁻⁵		WORK OF THE VENTRICLES AGAINST PRESSURE		
								Brachial artery			Pulmonary artery			Peripheral	Pulmonary	Left joules/min.	Right	
								syst.	diast.	mean	syst.	diast.	mean				joules/min.	joules/m ²
366	Rest	96	95	135	4.2	3.21	47	127	85	102	33	9	18	1500	318	61	11	8
	Sitting	100	—	177	4.8	3.68	52	87	60	73	31	11	20	1123	308	51	14	10
	Exercise	133	98	315	7.2	4.39	46	145	95	114	47	15	32	1476	362	94	26	18
371	Rest	108	95	132	3.5	3.67	58	132	06	112	16	6	9	1430	114	94	8	5
	Rest	111	97	147	3.1	4.59	71	118	84	102	13	6	0	1040	87	107	9	5
	Sitting	127	—	—	—	—	—	—	—	—	16	10	13	—	—	—	—	—
	Exercise	173	97	478	9.1	5.11	50	153	105	121	—	—	17	1110	159	141	20	12
372	Rest	93	96	142	3.2	4.44	71	114	63	88	16	4	9	1070	103	77	7	5
	Sitting	104	—	—	—	—	—	—	—	—	17	7	12	—	—	—	—	—
	Exercise	129	93	387	6.8	5.70	65	140	79	101	23	11	15	965	142	113	17	12
383	Rest	132	97	151	2.7	5.55	87	126	97	108	—	—	13	754	98	164	19	9
	Sitting	120	94	149	4.1	3.63	62	—	—	—	20	13	16	—	167	—	15	7
	Exercise	183	95	736	9.6	7.65	86	174	06	126	26	14	19	1356	04	263	39	19
	Rest	136	—	—	—	—	—	—	—	—	—	—	8	—	—	—	—	—
354	Rest	109	96	—	—	—	—	—	—	—	20	10	16	—	—	—	—	—
	Exercise	144	—	—	—	—	—	—	—	—	41	24	30	—	—	—	—	—
	Rest	141	93	—	—	—	—	—	—	—	32	11	20	—	—	—	—	—
368	Rest	83	93	142	4.1	3.48	69	124	73	92	24	4	13	1280	183	70	9	5
	Sitting	98	91	172	4.6	3.75	63	127	82	104	28	14	19	1350	240	85	15	9
	Exercise	159	86	983	—	—	—	199	111	148	59	27	43	—	—	—	—	—
358	Rest	79	91	177	3.6	4.97	120	120	60	86	33	17	23	722	182	109	30	16
	Sitting	88	—	183	5.3	3.50	76	124	60	89	32	16	23	1063	275	79	21	11
	Exercise	110	81	460	6.7	6.90	120	—	—	—	61	31	42	—	253	—	74	39
344	Rest	101	87	141	5.8	2.44	34	135	80	101	96	36	58	2330	1330	47	27	19
	Exercise	145	82	256	8.7	2.95	29	147	98	116	117	(50)	(70)	2210	(1330)	65	(39)	(28)

pulmonary artery. They were in no sense a homogeneous group, and the degree of functional disturbance varied widely. In table 3 it can be seen that the arterial oxyhemoglobin saturation in three patients (358, 344, and 368) was low at rest and decreased to a still lower level during exercise. With two exceptions (383 and 368) the severity of the work done by the patients was much less than that done by the normal subjects, as indicated by the rate of oxygen consumption.

Because of the reduced exercise tolerance of most of the patients, it was impossible to study them at higher work levels. In the patients with chronic pulmonary disease the stroke volume either remained unchanged or decreased during exercise. Six patients (366, 371, 372, 383, 354, 368) had pulmonary arterial pressures which were normal or close to normal at rest. In three of these (371, 372, 383) the increase in pulmonary arterial pressure during exercise was moderate. The three patients (358, 344, 368) who showed the most striking increase in pulmonary arterial pressure with exercise were the same ones whose oxygen saturation dropped sharply during exercise. Except in one case (371) there was an increase in both systolic and diastolic pressure in the pulmonary artery. In one patient (344) whose pulmonary arterial pressure was extremely high at rest, the pressure was recorded from the right ventricle but not from the pulmonary artery during exercise. Since the systolic pressures in the pulmonary artery and in the right ventricle are the same, the latter were used as a measure of pulmonary arterial systolic pressure. An estimated figure of 50 mm. Hg for the pulmonary arterial diastolic was assumed and this figure was used in the calculation of mean pressure, pulmonary vascular resistance and work of the right ventricle during exercise. In all the patients with chronic disease the pulmonary vascular resistance stayed the same or increased during exercise, in contrast to the normal subjects. During exercise the work of the right ventricle against pressure for a corresponding rate of oxygen consumption was invariably higher than in the normal subjects. In two patients (344 and 358) the discrepancy was very striking.

On the left side of figure 1 the patients' cardiac output values are plotted in relation to those of the normal subjects. Points falling to the left of the solid lines indicate higher than normal cardiac output, while those to the right indicate lower than normal output. One patient only (344) had a significantly low cardiac output and she had previously shown signs of congestive heart failure. On the right side of figure 1 all the patients' points fall to the right of the solid lines, indicating that the work of the right ventricle against pressure was abnormally high for the corresponding level of oxygen consumption. With one exception (383) the exercise performed by the patients was so light that the work of the right ventricle against pressure can be taken as an approximate measure of the total work of the right ventricle.

DISCUSSION

Comparison of the values obtained for cardiac output and oxygen consumption during exercise with those obtained by Bock, van Caulaert, Dill and others (4) shows a remarkable agreement. Their cardiac output method was a modification of the Haldane method, in which mixed venous carbon dioxide tension was estimated on the basis of an intrapulmonary equilibration procedure. For the corresponding rates of oxygen consumption, the values for two of our three normal subjects (377 and 378) invariably fall between the values of AVB and DBD. At the highest exercise level our third subject (379) corresponds to CVC. The data of Lindhard (3) obtained with the nitrous oxide method are also comparable. With normal subjects running on a treadmill, Dill, Edwards, and

Talbott (2) found the same relation of cardiac output to oxygen consumption as in the case of bicycle exercise. These data, too, correspond to ours. Stroke volume relationships show more variation because of the differences in pulse rate in response to a given amount of exercise.

Von Euler and Liljestrand (17) made observations on the pulmonary arterial blood pressure in the cat during exercise and found a moderate increase in all except one case. In a very recent note Hickam and Cargill (5) working with normal human subjects using the catheter technic report pulmonary arterial pressure essentially unchanged with increases in cardiac output up to nearly twice the resting level. The present study, undertaken without knowledge of their work, corroborates and extends this finding.

On the basis of the pulmonary arterial pressures found during exercise in normal individuals, certain changes in the pulmonary vascular bed during exercise may be postulated. If the dimensions of the pulmonary vascular bed remained unchanged during exercise, the mean pressure would rise in proportion to the increase in blood flow. The failure of the mean pressure to rise implies an expansion of the pulmonary vascular bed either by a widening of existing blood channels or by the opening of new ones or by a combination of both mechanisms. An actual decrease in pulmonary arterial pressure cannot be explained as a simple mechanical effect of increased blood flow. It suggests either that active vasodilatation occurs or that the increased negativity of intrathoracic pressure associated with deeper inspirations expands the vascular bed. The latter explanation is more consistent with current thoughts on the pulmonary circulation based on other work in this laboratory. Regardless of mechanism, there can be no doubt that a very significant expansion of the pulmonary vascular bed occurs as a normal response to exercise. If, as seems likely on hemodynamic grounds, more alveolar capillaries are opened up, the physiological advantages of such a vascular response would be seen not only in reduced work of the heart but also in increased efficiency of gaseous diffusion. This is in accord with the increased diffusion constant of the lung during exercise (18).

In studying patients with emphysema who had high pulmonary arterial pressure at rest, Hickam and Cargill (5) found a further large increase in pulmonary pressure with exercise. These findings, which are entirely comparable to those of the present study, are in striking contrast to the response to exercise of normal individuals.

Two factors are possibly of importance in explaining the rise in pulmonary arterial pressure with exercise in patients with chronic pulmonary disease. The first is the size and expansibility of the pulmonary vascular bed. Destruction of lung tissue, interstitial pulmonary fibrosis, or vascular sclerosis would be expected to reduce the number and cross section of the vessels and the expansibility of the vascular bed during increased blood flow.

The second factor is related to the effect of anoxia upon pulmonary vascular resistance. It may be seen in table 3 that the three patients whose arterial oxyhemoglobin saturation dropped significantly during exercise (358, 344, and 368) were the three whose pulmonary arterial pressures were highest during exer-

cise. Von Euler and Liljestrand's observations on cats (17) and the findings of Motley, Cournand, *et al.* (19) on human subjects indicate a sharp rise in pulmonary arterial pressure with acute anoxia produced by breathing a gas mixture low in oxygen. Might not the elevation of pulmonary arterial pressure occurring in some of our patients be related, at least in part, to the associated anoxia? In this respect it is interesting that von Euler and Liljestrand found a decrease in pulmonary arterial pressure with pure oxygen breathing to a level below that obtaining during room air breathing, suggesting that the slight degree of anoxemia normally present produces a slight increase in pulmonary vascular resistance. This argument tempts one to conclude that in subjects whose arterial blood is unsaturated at rest, a certain elevation of pulmonary arterial pressure above the normal may be due to the effects of anoxemia upon the size of the pulmonary vascular bed. However, since the evidence rests on acute experiments with high or low oxygen breathing, the application to chronic states of anoxia occurring in pulmonary disease can only be suggested as a possibility requiring further proof. In patients who have a marked decrease of oxygen saturation during exercise, the exercise may be considered as an acute experiment in anoxia, and it seems likely that the anoxemia may cause some of the increase in pulmonary arterial pressure.

The effects of chronic pulmonary disease upon the circulation may be considered from the over-all aspect of cardiac output or from the more restricted point of view of the work of the right ventricle. In figure 1 the data are plotted from both points of view. In this limited series of patients the cardiac output was maintained at an essentially normal level in all but the most extreme instance (344) even though a greater than normal work load was thereby placed upon the right ventricle. By increased work, the right ventricle compensated for the increased vascular resistance in the pulmonary circuit, and prevented a decrease in volume flow of blood.

In their revision of Bainbridge's monograph on "The Physiology of Muscular Exercise," Bock and Dill (1) state that "the heart, as a rule, reaches the limit of its powers earlier than the skeletal muscles, and its functional capacity determines a man's capability for exertion. . . . An exaggerated response on the part of the respiratory and circulatory systems involves a useless expenditure of energy; a deficient response rapidly throws out of gear the controlling influences of the nervous system, cripples the energies of the skeletal muscles, and renders exercise ineffective or even impossible." The latter situation is probably illustrated by one of our patients (344) whose cardiac output was significantly lower than normal both at rest and during exercise (fig. 1). She was able to perform only the mildest grade of work (oxygen consumption 256 ml. per minute per square meter of body surface) yet her right ventricle worked nearly three times as hard as the right ventricles of the normal subjects during heavy exercise. It seems probable that the functional capacity of the right ventricle determined this patient's capability for exertion. Another patient with increased pulmonary vascular resistance (358) was able to work nearly twice as hard in spite of a still greater work load on his right ventricle (fig. 1). Probably the greater functional

capacity of this man's right ventricle made possible his greater capacity for physical exercise.

These studies illustrate some of the difficulties involved in evaluating disability in chronic pulmonary disease. In most instances subjects are unable to exert themselves to a point where their cardiac output falls below the normal for that exercise level. Accordingly studies of the greater circulation reveal little. On the other hand studies of the pulmonary circulation during exercise reveal definite abnormalities in patients whose exercise tolerance is low. When considered in conjunction with other aspects of pulmonary function they extend the physiological evidence upon which an estimate of disability must be based.

SUMMARY

1. Studies of cardiac output and pulmonary arterial pressure were performed using the venous catheter technic in three normal individuals and in eight patients with various types of chronic pulmonary disease. Measurements were made at rest and during exercise on a stationary bicycle.

2. Two of the three normal subjects showed a decrease in the mean pressure in the pulmonary artery during exercise; all showed a marked drop in pulmonary vascular resistance and a minimal increase in the work of the right ventricle during exercise.

3. Three of the patients with chronic pulmonary disease showed a significant elevation of pulmonary arterial pressure at rest, and in all eight cases the mean pressure increased during exercise. There was either no change or an increase in the pulmonary vascular resistance during exercise, and the work of the right ventricle was invariably higher than in the normal subjects at a corresponding work level.

4. The findings indicate that the expansibility of the pulmonary vascular bed during exercise is limited in patients with chronic pulmonary disease.

5. Anoxia may contribute to the elevation of pulmonary arterial pressure during exercise in those patients whose arterial oxygen saturation falls.

6. The physiological evidence upon which an estimate of disability must be based in patients with chronic pulmonary disease is extended by studies of the pulmonary circulation during exercise.

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EFFECT OF BODY TEMPERATURE CHANGE ON THE CIRCULATION TIME IN THE CHICKEN¹

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Received for publication December 23, 1947

We have recently reported that an increase or decrease in the body temperature in the chicken (1) and in the turtle (2) causes a corresponding change in the arterial blood pressure. The mechanism of this relationship is in need of elucidation. Among those mechanisms which must be considered as possibly playing some rôle in this relationship are *a*) changes in the heart rate which are seen as the body temperature is varied; *b*) changes in the arteriolar and capillary responsiveness at various temperatures; and *c*) adjustments originating in the central nervous system which affect the tone of the blood vessels.

A few data which furnish partial answers to this question are already available. Thus the heart rate continues to increase during induced hyperthermia (1) even though there is usually no increase, and perhaps even a fall, in blood pressure; therefore the relationship is not entirely dependent on the changes occurring in heart rate. The peripheral arterioles show no observable changes in responsiveness with temperature change since there are no notable differences in the pressor response to epinephrine at various body temperatures (1). We have obtained evidence that the central nervous system plays an important rôle in the temperature-pressure relationship since destruction of the brain or sectioning of the cervical spinal cord in the turtle eliminates this relationship. This occurs even though the heart rate continues to change with changes in the body temperature (3).

In order to analyze the effect of body temperature change on the circulation, we felt that determination of the circulation time at various body temperatures might provide some insight into the mechanisms responsible for the observed blood pressure changes. Such information would also give presumptive evidence for changes in cardiac output.

METHODS

The chicken was used because the temperature-pressure relationship can be demonstrated most consistently in this animal. A total of 118 determinations of the circulation time was made on 12 chickens. Temperatures were measured with a mercury thermometer inserted deep into the cloaca. Blood pressures were recorded optically with the Hamilton manometer in the unanesthetized chicken according to the technique previously described by this laboratory (1).

¹ Aided by the A. D. Nast Fund for Cardiovascular Research, and in part by a grant from the War Department to the Michael Reese Hospital (Dr. L. N. Katz, responsible investigator). The department is supported in part by the Michael Reese Research Foundation.

The animals were cooled by packing them in crushed ice, and rewarmed by radiant heat (1).

The circulation time was estimated by the time of onset of the blood pressure response to the injection into the sciatic vein of 0.2 mgm. of acetylcholine dissolved in 0.2cc. saline. The circulation time was figured as the time from injection into the sciatic vein until the beginning of a marked fall in blood pressure.

In all 118 trials the blood pressure fell very significantly. The beginning of this pressure drop was considered to be the time required for circulation from the site of injection into the sciatic vein, through the heart and lung circuit, and to the peripheral arterioles. A significant slowing of the heart occurred in 81 of the 118 trials. However, this slowing usually occurred after the fall in systemic pressure.

RESULTS

1. *The normal circulation time of the chicken.* In our series of 12 animals the normal circulation time as determined by the fall in blood pressure averaged 2.8 seconds ranging from 1.3 to 5.0, with a standard deviation of 0.9 seconds. These

TABLE 1. AVERAGE CIRCULATION TIME AT VARIOUS BODY TEMPERATURES

BODY TEMPERATURE (°C)	AVERAGE CIRCULATION TIME Seconds
45	1.2
41.5	2.8
35	4.0
30	8.2
25	12.2

data are presented in figure 1 as points at 41–42°C. In those experiments in which the acetylcholine-induced bradycardia was observed, the time for slowing required an average of 4.5 seconds, about 1.7 seconds longer than the blood pressure effect.

2. *The effect of cooling.* In six chickens cooled from the normal body temperature of about 41.5° to about 25°C., the circulation time was increased from the normal of about 3 seconds to as much as 10 or 15 seconds at the lower temperature. This fall in the rate of circulation appeared to go step by step with the fall in body temperature, as indicated by table 1. Individual data are given in figure 1.

The time required for the onset of the bradycardia is also increased at lower temperatures so that at 25°C., the slowing of the heart was not apparent until 4 seconds or so after the fall in pressure, i. e., an average of about 16 seconds.

3. *The effect of warming.* In six chickens warmed from the normal body temperature to as high as 47°C. the rate of circulation continued to increase, with the circulation time values as low as 0.6 seconds (fig. 1). This reduction in the circulation time occurred despite the fact that the blood pressure almost always had begun to fall by the time the body temperature has been raised to

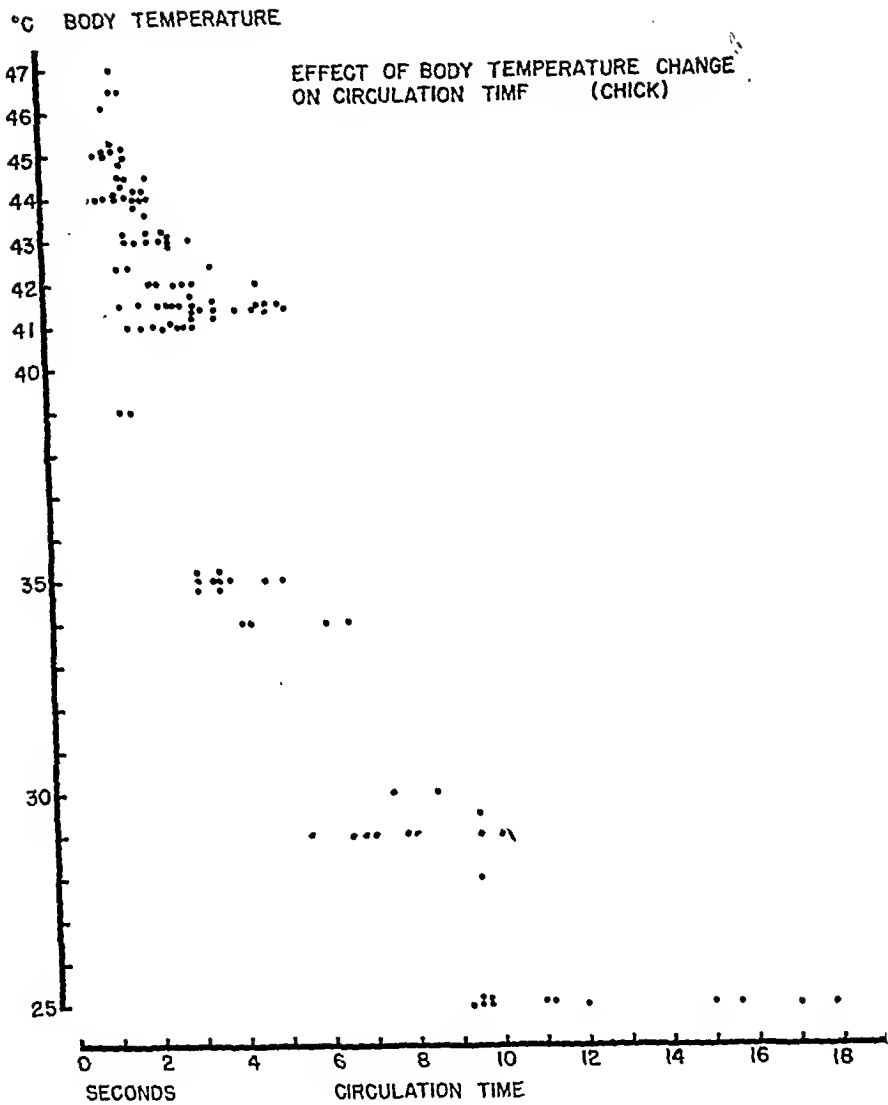


FIG. 1.

TABLE 2. RESPONSE OF BLOOD PRESSURE TO ACETYLCHOLINE INJECTIONS AT VARIOUS BODY TEMPERATURES

BODY TEMPERATURE (°C)	ARTERIAL PRESSURE	
	Pre-injection (mm. Hg)	Average minimal pressure observed mm. Hg
46	50	30
45	83	30
44	103	39
43	107	36
41.5	110	43
35	105	46
30	99	47
25	59	35

about 44°C. The heart rate also continued to increase with increasing temperatures, as previously noted (1). The bradycardia induced by acetylcholine occurred about 0.5 seconds after the depressor effect, e. g., a total time of about 1.2 seconds at 46°C.

4. *The degree of blood pressure fall.* The injection of acetylcholine lowered the pressure momentarily to levels averaging about 40 mm. Hg. The minimal level obtained was not dependent on the pre-injection level, nor was there any consistent change in the degree of the blood pressure fall as the animals were cooled or warmed (table 2.).

DISCUSSION

The normal circulation time in the chicken, 3 seconds, is quite rapid when compared with that observed with the same technique in the dog (4) or in man (5). This is probably due in part to the small size of the chicken, which reduces the length of the mean path from the point of injection to the site of action. It may also be related to the higher body temperature of this animal, and the consequent higher metabolic rate, requiring a greater circulation.

The finding that the circulation is slowed as the body temperature falls is in accord with data which we have obtained on blood pressure, heart rate (1), and with the results of Randall on respiration (6). When the animal is cooled, the reduced rate of circulation of the blood is apparently sufficient to nourish the tissues at the lower rates of metabolism which must obtain at the lower body temperatures. This may be inferred from the fact that no apparent injury occurs to the animal when, after a period of two hours at body temperatures as low as 25°C., it is rewarmed to its normal body temperature. As has been noted, the animals show no signs of shock, appear normal and resume feeding. These circumstances demonstrate that no serious disparity exists between the metabolic demands of the tissues for oxygen and substrate, and the rate of delivery of these substances to the tissues by the blood stream.

It is apparent that the circulation speeds up (decreased circulation time) as the body temperature is increased above normal and that it continues to do so practically until the point of death (7, 8). This occurs despite the fact that the blood pressure is not necessarily increased in experimental hyperthermia and that it may even fall to very low levels (65/50 mm. Hg). These results show that the circulation time is a function of the metabolic needs of the tissues rather than of the arterial blood pressure. Since the metabolic needs of the tissues depend in large part upon their temperatures, it would appear that each tissue autonomously determines the volume of blood which traverses its blood vessels.

It is of some interest that in hyperthermia in which the circulation is speeded up and the blood vessels of the tissues must be somewhat dilated to permit a greater perfusion of blood, a maximal dilatation is not obtained even at high temperatures (46°C.) since a fall in blood pressure still occurs on the injection of acetylcholine. Thus the vessels are still partially constricted under these extreme conditions.

The progressive rise in heart rate with increasing body temperatures would also appear to be an autonomous tissue function, in this case of the heart itself. It is possible that during the tachycardia seen in hyperthermia with rates of as much as 540 per minute, the heart may reach the limit of effective cardiac output and begin to show signs of failure, and this possibly may play a rôle in the death of the animals.

An unexpected finding was that the time required for the fall in pressure by action of acetylcholine on the pacemaker of the heart was considerably longer than that required for the fall in blood pressure due to the peripheral action of the drug on the arterioles. The reason for the cardiac delay is not apparent at present.

Our present data support our earlier studies in which we showed no significant change in responsiveness to epinephrine of the blood vessels at various temperatures (1). The present results are similar in that cooling or warming of the blood vessels did not significantly alter the degree of the depressor response to acetylcholine.

The reduced rate of blood flow during cooling would appear to be related not only to the greatly reduced heart rate but also to the very likely reduction in minute cardiac output. Both factors would tend to reduce the blood pressure. The likely reduction of blood vessel calibre under hypothermic conditions would tend to raise the blood pressure by increasing resistance to flow. The resolution of these conflicting trends may be a simple algebraic summation.

In hyperthermic states the dilatation produced by the increased demand for blood by the tissues would tend to cause a fall in blood pressure. The increased rate of return of blood from the tissues and the consequent increased cardiac output would tend to raise the blood pressure. However, at temperatures within two or three degrees above normal, the blood pressure regulating mechanisms appear to maintain a relatively constant level.

The regulation of the blood pressure thus appears to depend on factors other than the changes in heart rate, the circulation time or the rate of metabolism of the peripheral tissues. The blood vessels show no change in responsiveness at various body temperatures to either vasopressor and vasodepressor drugs. Instead, the mechanisms for blood pressure regulation appear to reside primarily in the central nervous system. It is these central nervous mechanisms which eventually fail as the body temperature is raised sufficiently above normal. The temperature-pressure mechanism, which functions to permit an increased perfusion of the tissues at higher temperatures, is apparently finally overwhelmed by the increasing vasodilatation in the periphery, and by approaching the limit of adequate function of the heart at high rates. Our data therefore suggest that the final failure of the circulation in extreme hyperthermia lies not only in the peripheral vessels which are dilated, but still responsive even at the extremes of body temperature, not in the ability of the heart to pump blood, but actually it depends to a very large degree upon the failure of the central nervous mechanism which acts to regulate the arterial blood pressure.

SUMMARY

1. The acetylcholine circulation time of the chicken at normal body temperatures (41.5°C.) is about 2.8 seconds.

2. When the body temperature of the animal is lowered by application of ice packs, the circulation time is increased. Increasing the body temperature with radiant heat decreases the circulation time.

3. The circulation time is not necessarily related to changes in the blood pressure which occur as the temperature of the animal is changed, but appears to be due primarily to changes in the peripheral tissues.

4. A discrepancy between the time required for the action of acetylcholine on the peripheral blood vessels and on the rate of the heart is noted.

5. Some aspects of these data are discussed in terms of the body temperature-blood pressure relationship.

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QUANTITATIVE STUDIES ON THE COMPARATIVE ACTIVITY OF CALCIUM AND CHEMICALLY RELATED IONS ON THE COAGULATION OF BLOOD¹

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Received for publication January 6, 1948

A direct quantitative study of the influence of varying the concentration of calcium on the coagulation time of blood was not possible until Steinberg (1) discovered that a phenol-formaldehyde resin, Amberlite IR-100, completely removed calcium without inducing any other demonstrable changes in the blood. By using chloro-methyl-silane (Silicone) coated glassware with Amberlite and by taking precautions to prevent contamination of the blood with tissue juice, the lysis of platelets is almost entirely prevented and it seems reasonable to assume that the blood obtained is practically identical with the circulating blood except for the absence of calcium. The effect of different concentrations of calcium on the clotting of blood and plasma can therefore be determined simply and with precision by the direct addition of known amounts of calcium salts. This procedure is likewise suitable for studying the action of related ions, particularly strontium, magnesium and barium.

In this paper the results of such study are reported. The influence of different concentrations of calcium, strontium, magnesium and barium on the clotting time of whole blood and plasma and prothrombin time are also considered in man, dog and rabbit. Consideration is also given to the possibility of an antagonism between calcium and related bivalent ions in the process of coagulation.

EXPERIMENTAL.

Blood was obtained by venipuncture in man and dog and from the main artery of the ear in rabbits. Precaution was taken to avoid injuring tissue and to prevent foaming. Needles, syringes and collecting tubes used were coated with chloro-methyl-silane. The blood was immediately passed through purified Amberlite IR-100 according to the technique recently described (2). The collecting tube and the syringe were chilled and the decalcified blood kept in an ice bath until used.

To remove traces of calcium from the thromboplastin, sodium oxalate (0.1 ml. of 0.1 M sodium oxalate per rabbit brain) was added before triturating the brain with acetone. The chlorides of calcium, strontium, magnesium and barium were used, and their solutions were made isotonic to blood and then diluted to the desired strength by mixing with physiological sodium chloride solution; this was

¹ This work was supported by a grant from the U. S. Public Health Service.

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done to eliminate any possible influence of osmotic pressure changes. The coagulation time was determined by adding to 0.1 ml. of calcium chloride (or other salt studied), 0.4 ml. of blood or plasma decalcified with Amberlite. Pyrex tubes with an internal diameter of 11 mm. were used. All observations were carried out at 37°C.

The effect of varying concentrations of calcium and strontium chloride on the coagulation time of blood and plasma decalcified with Amberlite. From the results recorded in table 1 it will be observed that the shortest coagulation time for human, dog and rabbit blood is not obtained until the concentration of calcium

TABLE 1. INFLUENCE OF THE CONCENTRATION OF CALCIUM AND STRONTIUM ON THE COAGULATION TIME OF BLOOD DECALCIFIED WITH AMBERLITE¹

CONCENTRATION OF THE SALT ²	COAGULATION TIME					
	CaCl ₂			SrCl ₂		
	Human blood	Dog blood	Rabbit blood	Human blood	Dog blood	Rabbit blood
<i>M</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
0.0002	>60	>60	>60			
0.0005	8 $\frac{3}{4}$	29	27 $\frac{1}{2}$			
0.001	6	8 $\frac{1}{2}$	8 $\frac{1}{2}$			
0.0015	5 $\frac{3}{4}$	4 $\frac{1}{4}$	5 $\frac{1}{2}$	>60	>60	>60
0.002	5 $\frac{3}{4}$	4	5 $\frac{1}{2}$	28 ³	>60	>60
0.004	5 $\frac{1}{2}$	3 $\frac{1}{2}$	5 $\frac{1}{4}$	12 $\frac{3}{4}$	19	17 $\frac{1}{2}$
0.006	6 $\frac{3}{4}$	4 $\frac{1}{2}$	8	11 $\frac{1}{2}$	15 $\frac{1}{2}$	12 $\frac{1}{2}$
0.008	9	5	9 $\frac{1}{4}$	11	13	12 $\frac{1}{4}$
0.01	9	5	9 $\frac{1}{2}$	18 $\frac{3}{4}$	17	17
0.012	10 $\frac{1}{4}$	6 $\frac{1}{4}$	10 $\frac{1}{4}$	28	39	36
0.0135	11	6 $\frac{1}{4}$	34	34	58 ³	>60
0.0155	11 $\frac{1}{2}$	10 $\frac{1}{2}$	47	59 ³	>60	
0.0175	11 $\frac{3}{4}$	10 $\frac{1}{2}$	>60	>60		
0.018	13 $\frac{1}{2}$	21				
0.02	14 $\frac{1}{2}$	37				

¹ To four volumes of blood treated with Amberlite one volume of CaCl₂ or SrCl₂ solution was added. Isotonic solutions of these salts were prepared and diluted to the desired concentration by mixing with physiological sodium chloride solution.

² The molarity is calculated on basis of volume of blood.

³ Only a few threads of fibrin formed.

chloride is approximately 0.0015 M. This concentration is in the range of the normal calcium level of the blood, which is also about 0.0015 M. When the calcium concentration is reduced below one half of the normal, a definite delay in coagulation occurs. A minimum coagulation time is obtained over a wide range of calcium concentrations, but above 0.004 M inhibition occurs in human, dog and rabbit blood, becoming more evident as the concentration of the salt is progressively increased. Oddly, the depressing action of calcium is most marked in rabbit blood. Coagulation of plasma (table 2) follows essentially the same pattern except that the clotting time is shorter. This can be explained, at least in part, by the earlier detection of the clot in plasma than in whole blood.

Strontium has a much weaker clotting activity than calcium. The minimal level required for optimum or shortest coagulation of whole blood and plasma is definitely higher (0.006 to 0.008 M for both whole blood and plasma of man, dog and rabbit). The range of optimal concentrations at which most rapid clotting occurs is also considerably more narrow than in the case of calcium since inhibition already occurs at a level of 0.01 M (tables 1 and 2).

Magnesium is incapable of causing coagulation of either whole blood or plasma decalcified with Amberlite at any concentration. In the presence of thrombo-

TABLE 2. INFLUENCE OF THE CONCENTRATION OF CALCIUM AND STRONTIUM ON THE COAGULATION TIME OF PLASMA DECALCIFIED WITH AMBERLITE¹

CONCENTRATION OF THE SALT ²	COAGULATION TIME					
	CaCl ₂			SrCl ₂		
	Human plasma	Dog plasma	Rabbit plasma	Human plasma	Rabbit plasma	Dog plasma
M	min.	min.	min.	min.	min.	min.
0.0002	>60	>60	>60			
0.0005	7½	12	15½			
0.001	4½	7½	9½			
0.0015	3½	3¾	4	>60	>60	>60
0.002	2½	2½	2¾	28	45	>60
0.004	2	2	2½	8¾	23	29½
0.006	2½	2¾	2½	7	18½	21
0.008	3	3½	2¾	8½	9¾	11¾
0.01	3½	4	3	8½	11	13
0.012	5¾	6¼	6¾	8¾	15	20¾
0.0135	9	7¾	7¾	10¼	27	45
0.0155	9¼	9¾	9½	15	38	54
0.0175	19	17	17	17	58 ³	>60
0.018	21	19	22	>60	>60	
0.02	38	22	24			

¹ To four volumes of plasma decalcified with Amberlite one volume of CaCl₂ or SrCl₂ solution was added. Isotonic solutions of these salts were prepared and diluted to the desired strength by mixing with physiological sodium chloride solution.

² The molarity is calculated on basis of volume of plasma.

³ Only a few threads of fibrin formed.

plastin, however, a weak clotting activity can be demonstrated, as will be described later. Barium is found to possess no demonstrable coagulant activity, even in the presence of thromboplastin.

The comparative inhibitory action of strontium, magnesium and barium on coagulation when an optimum concentration of calcium is present. A fixed amount of calcium (0.005 M CaCl₂), mixed with varying concentrations of SrCl₂, MgCl₂, and BaCl₂, was added to decalcified plasma and the coagulation time determined. The results are summarized in table 3. It will be noted that the retarding effect of SrCl₂ on clotting is less marked when calcium is present and that strontium and magnesium present essentially the same inhibitory activity. This inhibitory effect is shown not only by the delayed coagulation but also by the character of the

clot, which becomes softer, poorer in texture and ultimately very incomplete and almost granular. Barium, at a relatively low concentration, causes complete

TABLE 3. EFFECT OF DIFFERENT CONCENTRATIONS OF STRONTIUM, MAGNESIUM AND BARIUM ON THE COAGULATION TIME OF PLASMA DECALCIFIED WITH AMBERLITE IN THE PRESENCE OF AN OPTIMUM CONCENTRATION OF CALCIUM CHLORIDE (0.005 M)

CONCENTRATION OF THE SALT ¹	COAGULATION TIME					
	SrCl ₂		MgCl ₂		BaCl ₂	
	Human plasma	Rabbit plasma	Human plasma	Rabbit plasma	Human plasma	Rabbit plasma
<i>M</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
0.0004	2	2 $\frac{3}{4}$	2	2 $\frac{1}{4}$	2	2 $\frac{3}{4}$
0.0008	2 $\frac{1}{4}$	3	2	2 $\frac{1}{4}$	2 $\frac{1}{4}$	2 $\frac{3}{4}$
0.002	2 $\frac{1}{2}$	3 $\frac{1}{4}$	2	2 $\frac{3}{4}$	2	3
0.004	3	3 $\frac{1}{2}$	3	5 $\frac{1}{4}$	3 $\frac{1}{4}$	4 $\frac{1}{2}$
0.007	3 $\frac{3}{4}$	4 $\frac{1}{4}$	4 $\frac{1}{4}$	8	5 $\frac{1}{4}$	6 $\frac{1}{2}$
0.01	4 $\frac{1}{2}$	5	8 $\frac{1}{2}$	9	23	28
0.013	6 $\frac{1}{4}$	7 $\frac{1}{4}$	10 $\frac{1}{4}$	10	∞	∞
0.017	10 $\frac{1}{4}$	11	11 $\frac{3}{4}$	11 $\frac{3}{4}$		
0.02	12 $\frac{1}{2}$	13 $\frac{1}{2}$	17	13 $\frac{1}{4}$		
0.023	18	18	17 $\frac{1}{2}$	19		
0.027	34	39	38	45		
0.03	∞	∞	∞	∞		

¹ The concentration resulting when one volume of the salt solution was mixed with four volumes of plasma. To the salt was added the calculated amount of CaCl₂ to have a concentration of 0.005 M in the final mixture.

TABLE 4. INFLUENCE OF VARYING CONCENTRATIONS OF CALCIUM, STRONTIUM AND MAGNESIUM ON THE PROTHROMBIN TIME OF PLASMA DECALCIFIED WITH AMBERLITE

CONCENTRA- TION OF THE SALT	PROTHROMBIN TIME								
	CaCl ₂			SrCl ₂			MgCl ₂		
	Man	Dog	Rabbit	Man	Dog	Rabbit	Man	Dog	Rabbit
<i>M</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
0.06	18	12	13	∞	∞	∞	∞	∞	∞
0.05	16.5	10.5	11	1620	680	750	∞	∞	∞
0.04	15	8.5	9	36	14.5	14.5	293	174	182
0.03	13.5	8	8.5	28	13.5	13.5	125	81.5	89
0.02	13	7.5	7.5	22	13	13	78	46	43
0.01	12	7.5	7	19.5	12.5	12	64	35.5	31.5
0.005	12	7	7	22	12.5	12	58	32.5	30
0.0025	12	7	6.5	30	18.5	16	69.5	37	38
0.00125	13.5	7	7	37	21	21	74.5	46	45.5
0.0006	15	8	8.5	55	36	33	89	58	57
0.0005	19	8.5	10	83	39	37	∞	∞	∞

incoagulability of the blood. The barium ion, it should be pointed out, precipitates plasma proteins similar to other heavy metals and this probably ex-

plains its marked inhibitory effect on coagulation. By using fixed concentrations of strontium, magnesium and barium but varying strengths of CaCl_2 the characteristic wide range of the concentrations of calcium capable of causing an optimum clotting of plasma tends to be more narrow.

The comparative activity of calcium, strontium and magnesium on the prothrombin time. From the results of table 4 one can see that calcium is much more active than strontium but that, surprisingly, even magnesium has a definite even though weak activity. The range of concentrations at which a minimum prothrombin time is obtained is widest for calcium. Significantly, however, a solution as diluted as 0.0006 M MgCl_2 will produce coagulation when excess thromboplastin is present. The optimum concentration of strontium and magnesium is essentially the same, but the inhibitory action of magnesium appears to be greater.

TABLE 5. INFLUENCE OF VARYING CONCENTRATIONS OF STRONTIUM, MAGNESIUM AND BARIUM IN PRESENCE OF A FIXED OPTIMUM AMOUNT OF CALCIUM (0.005 M) ON THE PROTHROMBIN TIME OF PLASMA DECALCIFIED WITH AMBERLITE

CONCENTRATION OF THE SALT	PROTHROMBIN TIME					
	SrCl_2		MgCl_2		BaCl_2	
	Man	Rabbit	Man	Rabbit	Man	Rabbit
<i>M</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
0.006	12	6.5	12	6.5	12	6.5
0.012	12	6.5	12	6.5	13	7
0.018	12	6	12	6.5	14.5	8.5
0.024	12	6.5	12.5	6.5	17	9.5
0.042	12	6.5	12.5	6.5	19.5	10.5
0.06	15.5	9	14.5	9.5	26.5	15
0.078	25	15	26.5	14	∞	∞
0.102	35	20.5	38	22.5		
0.12	43	28	40.5	26.5		
0.138	114	48	98	42.5		
0.16	165	112	124	79.5		
0.18	302	148	256	124		
0.2	∞	∞	∞	∞		

To test whether this action is real or apparent, the prothrombin time was determined using a fixed optimum concentration of calcium and the same series of dilutions of strontium and magnesium chloride. From the results in table 5 it is obvious that the inhibitory action of strontium and magnesium is practically equal and of the same order of magnitude as the retarding action of calcium itself.

DISCUSSION

It can be concluded that the minimum amount of calcium required for optimum coagulation, as judged by the shortest clotting time, is approximately the same as the concentration normally found in blood. Clotting still occurs, however, at much lower concentrations especially if the amount of thromboplastin present is increased. This possibly explains why Vines (3) found that only one seventeenth

of the serum calcium was necessary for coagulation and Stassano and Daumas (4) likewise stated that only 13 to 21 mgm. of calcium per liter of blood were required for clotting. It should be emphasized that the coagulation time as determined ordinarily is influenced mainly by the variations of the available thromboplastin and that the changes in calcium concentration which can occur in physiological or pathological conditions are probably never sufficient to affect the speed of coagulation.

While it is generally accepted that calcium is necessary for the conversion of prothrombin to thrombin, it is still a moot question whether it acts catalytically or stoichiometrically. The former view has been accepted almost unhesitatingly, but the senior author has recently demonstrated (5) that if the concentration of calcium is progressively diminished a point is reached at which the prothrombin conversion is decreased even though excess thromboplastin is present, which clearly suggests a stoichiometric relationship. Our finding that the coagulation begins to be retarded when the calcium concentration is reduced a little below 50 per cent of the normal is likewise in accord with the stoichiometric view.

The possibility that calcium may have a second rôle in coagulation must, in the light of recent developments, be seriously considered. Ferguson (6), in 1934, reported that calcium is necessary for the agglutination and lysis of platelets. Recently the senior author obtained data (7) showing that thromboplastin occurs in the plasma in the form of a precursor which he designated as thromboplastinogen. For the activation of the latter a factor present in platelets is required. A little later, Milstone (8) also recognized that plasma contains a precursor of thromboplastin, which he has named prothrombinokinase. He has reported experiments indicating that calcium is necessary for the conversion of the precursor to active thromboplastin. Since there are findings which indicate that thrombin is necessary for the agglutination of platelets (9) it remains problematic whether calcium functions only in thrombin formation or also in platelets lysis and in the activation of the thromboplastin precursor.

Whether calcium can be replaced by other bivalent ions has been investigated by various authors. It was observed by Arthus and Pagès (10) that strontium had coagulant action, but was less active than calcium. Similar observations were recorded by Heard (11), Ringer and Sainsbury (12) and Mellanby (13). The last three authors claimed that barium chloride also possessed clotting activity, while Arthus and Pagès had found it ineffective. Loomis and Seegers (14) recently found that strontium was a fair but rather slower activator of prothrombin than calcium and that even magnesium and barium had activity, though very weak. Richards and Johnson (15) studied the substitution of strontium for calcium in the one-stage prothrombin method and found that the prothrombin time was much longer and higher concentrations of strontium were required as compared with calcium for optimal activity. The findings of the present investigation confirm the previous observations that strontium has definite but weaker action than calcium in coagulation. Magnesium is ineffective to bring about clotting of decalcified blood and plasma unless a high concentration of thromboplastin is present. It appears, therefore, that the action of calcium in coagulation is not specific, but that strontium and magnesium likewise show this action.

Calcium and also strontium and magnesium exert an inhibitory action on clotting as their concentration is increased above the optimum level. This was observed as early as 1896 by Horne (16) and recently confirmed by Greville and Lehmann (17) who also concluded from their studies that a true antagonism exists between Mg^{++} and Ca^{++} in respect to plasma clotting. Since these ions accelerate or retard clotting purely on the base of their concentration, the coagulation time or prothrombin time is therefore a resultant of a positive or activating and a negative or inhibitory action of calcium and related bivalent ions. Since the activating effect of strontium and magnesium is relatively weak, the inhibitory influence becomes very evident. If the activating action is made constant by selecting, for instance, an optimal concentration of calcium, it is found that the inhibitory effect of calcium, strontium and magnesium is essentially the same and is relatively weak.

It seems logical to assume that the activating effect of calcium, strontium and magnesium takes place directly in the interaction of prothrombin with thromboplastin. The possibility that they may also function in the lysis of platelets must not be ignored. On the other hand, the inhibitory action may perhaps be due to the ability of these ions to stabilize proteins, particularly fibrinogen. It should also be emphasized that extrinsic factors may alter the relative domination of one effect over the other. Thus, in the coagulation of whole blood, the activating power of calcium, strontium and magnesium is comparatively weak due to the limited quantity of thromboplastin available. When excess of thromboplastin is present, then the activating force overshadows the inhibitory and under these conditions magnesium exhibits clotting activity. From the results presented one can find no evidence of a direct antagonism between calcium and the other ions.

SUMMARY

By studying the effect of varying concentrations of calcium chloride added to blood after decalcification by means of Amberlite, it was found that the optimal amount of calcium is approximately the same as normally present in blood. Strontium has a weak clotting activity and a higher molar concentration is required. Magnesium fails to clot decalcified blood.

Calcium, strontium, magnesium and barium have an inhibitory action on coagulation which becomes greater as their concentration is increased above the optimum level. By adding a fixed optimum concentration of calcium and varying amounts of strontium, magnesium and barium to decalcified plasma it was found that the first two have about the same inhibitory action, whereas barium has a much greater anticoagulant effect.

Strontium is decidedly less active in coagulation than calcium in the presence of excess thromboplastin. Magnesium has a weak but definite activity. The molar concentrations required for the shortest prothrombin time are higher for strontium and magnesium than for calcium. With a fixed optimum concentration of calcium the inhibitory effect of strontium and magnesium is found to be approximately the same and of about the same magnitude as that of calcium.

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LIMITATIONS OF THE RENIN-HYPERTENSIN HYPOTHESIS¹

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Received for publication December 19, 1947

The discovery of the action of renin on the serum globulin hypertensinogen and the separation of the polypeptide hypertensin (angiotonin 1, 2) led to the hypothesis that this transformation is enzymatic and that the resulting elevation in blood pressure is a function of newly formed hypertensin. Increased renin activity, it was postulated would result in larger hypertensin production.

Recent studies (3-8) necessitate re-evaluation of the renin-hypertensin theory as an explanation of the humoral mechanism concerned in hypertension. Attempts to demonstrate adequate amounts of renin in the blood of hypertensive patients and animals so far have been unsuccessful. Moreover, it has not been possible to find hypertensin in the blood of hypertensive patients or animals (9) or to detect it in plasma withdrawn at the peak of the pressure curve subsequent to an injection of renin into rabbits (10).

The study that follows supplies further evidence that the blood pressure response to renin cannot be explained adequately by the production of hypertensin.

MATERIALS AND METHODS

Renin preparations free of hypertensinase² were made following the method described by Katz and Goldblatt (11) as modified by Dexter (3). Fresh hog kidneys in 5-kgm. batches were prepared in an electric meat grinder and, after the addition of twice their weight of distilled water and thorough mixing, were allowed to stand overnight at 5°C. The material was then strained through five thicknesses of gauze and the pulp discarded. The resulting kidney extract was brought to pH $2.9 \pm .05$ at 0°C. with 10 per cent trichloroacetic acid. Cold saturated NaCl solution was added until the molar concentration was .92 to precipitate the hypertensinase. The 'brei' was then filtered over a period of 5 to 6 hours at +5°C. The resulting clear, yellow filtrate was adjusted to pH 5.0 with 1N NaOH; 400 grams of solid ammonium sulfate per liter of filtrate were added and after 4 or 5 hours it was filtered through large Buchner funnels with E & D filter paper # 615 and Kieselguhr. The precipitate was redissolved in a small amount of distilled water; it was then dialyzed against distilled water until the dialysate was free of sulfate and the resulting renin solution was lyophilized. This method yielded about three grams of protein per 5-kgm. batch of kidney. Two mgm. were equivalent to one Swingle Dog Unit (12) on direct

¹ Aided by a grant from the Commonwealth Fund.

² Grateful acknowledgement is made to Dr. Dexter for his contribution of hypertensinogen and for confirming that the renin preparation was free of hypertensinase.

injection. Hypertensinogen furnished by Dr. Dexter was used in the first experiments. Later, Armour Bovine fraction IV_1 was secured. In some experiments freshly prepared dog serum freed of hypertensinase was substituted for hypertensinogen.

Hypertensinogen and renin solutions kept in motion by an electric stirrer were incubated in a water bath at 38°C . Two cc. of $1/15\text{ N}$ phosphate buffer (pH 7.5) were added for every 10 cc. of 1 per cent hypertensinogen solution to maintain a pH of 7.4 to 7.5.

In the experiments with short incubation times, great care was taken to stop the hypertensin formation abruptly. The interruption was accomplished by adding a previously determined amount of 1N HCl to bring the pH to 5.2. The acidified fluid was then immersed in boiling water. Following deproteinization and removal of the precipitate (moist Whatman $\#1$), the filtrate was lyophilized, the dry product, redissolved in distilled water, was tested for its hypertensin content within the hour by intravenous injection into the foreleg of a nembutalized dog. The femoral artery of such an animal was cannulated and connected with a mercury manometer which recorded the blood pressure on a kymograph. The direct renin assay was carried out in the same way. The technique for perfusion of the rabbit ear has been described (13).

RESULTS

Speed of hypertensin formation. In the course of many experiments it was observed that the average latent period following intravenous injection of renin into the foreleg of a dog is 16 seconds. This accords with values reported in the literature (14). Hypertensin formation obviously must be prompt to be causatively associated with the rise in pressure. The velocity of the catalytic process *in vitro* is illustrated in the following typical experiments, nos. 1 to 4 (table 1).

As is evident, large amounts of hypertensin were formed after only 20 or 30 seconds incubation of renin with hypertensinogen or serum.

Completeness of transformation of hypertensinogen. Interestingly enough prolongation of the incubation of renin and hypertensinogen to one and one half or three minutes did not increase the hypertensin yield significantly. This is demonstrated in table 1, experiments 5 to 9, and by figures 1, 2, 3 and 4.

The results of the experiments 6, 7, 8 and 9 indicate that 20 to 30 seconds of incubation of 100 cc. of hypertensinogen or serum with 1.5 to 2.5 units of renin were sufficient to result in the total amount of hypertensin the available hypertensinogen is able to yield. The amount of fraction IV_1 (one gram) used in these experiments compares satisfactorily with the possible total quantity of this fraction present in the plasma of a small dog³.

It can be assumed, therefore, that injection of sufficient renin into the blood stream of a dog rapidly catalyzes the formation of the total possible amount of hypertensin.

³ The total plasma proteins of a 5.5 kgm. dog (450 cc. blood, 250 cc. plasma) approximate 14 grams. The α -globulin fraction comprises about one-fourteenth of the total plasma proteins. (E. J. Cohn *et al.*, J. Am. Chem. Soc. 68: 459, 1946.)

Action of increasing amounts of renin in vitro and in vivo. As has been reported (14) the duration of the blood pressure elevation following hypertensin injection is not more than 2.5 to 3 minutes. In contrast, an injection of only 2 or 3 dog units of renin is followed by a sustained elevation of the blood pressure extending to 20 and sometimes even exceeding 30 minutes. The differences in the two

TABLE 1

EXPERIMENT	RENIN UNITS	1% HYPERTENSINO- GEN (H) OR DOG SERUM (S)	INCUBATION PERIOD	BLOOD PRESSURE RISE	WEIGHT OF DOG
		cc.	sec.	mm. Hg.	kgm.
1 ¹	1.5	100 H	30	42	10
2	1.5	100 H	30	71	7.7
3	2.5	100 S	30	86	8
4	2.5	100 S	20	85	7.6
			min.		
5 ²	1.5	100 H	3	58	10
	1.5	100 H	1½	52	
	1.5	100 H	½	42	
6	1.5	100 H	3	73	7.7
	1.5	100 H	1½	74	
	1.5	100 H	½	71	
7	2.5	100 S	3	90	8
	2.5	100 S	½	86	
8	2.5	100 S	1½	70	9
	2.5	100 S	½	74	
9	2.5	100 S	3	85	7.6
	2.5	100 S	½	81	
10 ³	2	20 H	5	58	7
	8	20 H	5	54	
11	.5	140 H	10	78	6.8
	1	140 H	10	76	
	2	140 H	10	80	
12	1	100 S	5	68	6.8
	3	100 S	5	74	
	9	100 S	5	78	

¹ Hypertensin yield after 20 to 30 seconds incubation of renin with hypertensinogen or serum (experiments 1 to 4).

² Completeness of the hypertensin yield after only 20 to 30 seconds incubation of renin with hypertensinogen or serum (experiments 5 to 9).

³ Relation of hypertensin yield to quantity of renin (experiments 10 to 12).

pressor curves are striking (fig. 5). Slow and continuous hypertensin formation has been suggested as the cause of the prolonged elevation of the blood pressure following renin (14). There is no evidence to support this or that there may be delay in the in vivo mixing of the hypertensinogen and renin, two possibilities that should be borne in mind. The observed rapid completion of hypertensin formation in vitro and the absence of information to the contrary in the living animal is noteworthy negative evidence.

On the other hand, the speed and completeness of the *in vitro* transformation of hypertensinogen probably is an expression of excess renin. This was obtained with 1.5 Swingle units as shown in experiments 5 and 6 and 2.5 units in experiments 7, 8 and 9 (table 1). If these amounts of renin represent excess, increasing quantities should not lead to greater hypertensin yield. That this is the fact was demonstrated in another series of experiments, nos. 10 to 12 (table 1).

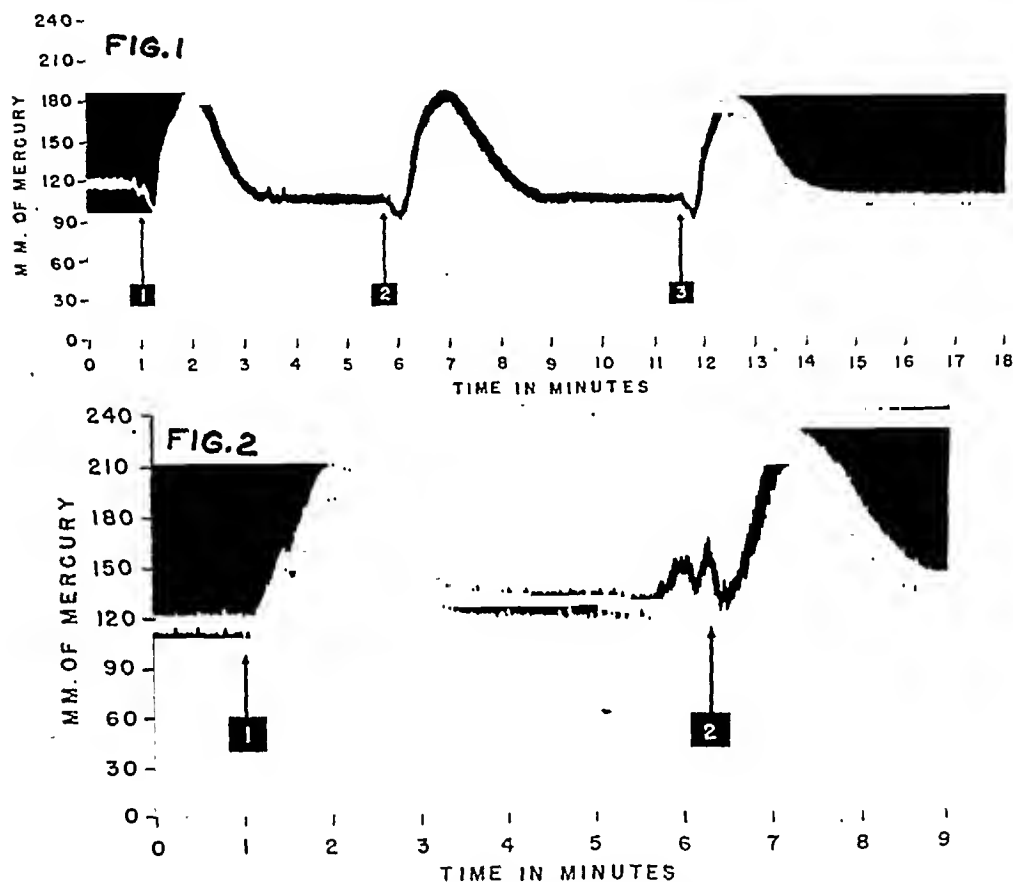


FIG. 1. BLOOD PRESSURE CURVES FOLLOWING I.V. INJECTION OF HYPERTENSIN produced by incubating 1.5 units of renin with 100 cc. of 1 per cent hypertensinogen for: (1) three minutes, (2) one and one-half minutes, (3) one-half minute (Exp. 6, table 1).

FIG. 2. BLOOD PRESSURE CURVES FOLLOWING I.V. INJECTION OF HYPERTENSIN produced by incubating 2.5 units of renin with 100 cc. of dog serum for: (1) one-half minute, (2) three minutes (Exp. 7, table 1).

No significant differences in hypertensin yield were observed with quadrupled renin amounts (experiments 10 and 11). Nine Swingle units of renin yielded almost the same amount of hypertensin as three, and three units only insignificantly more than one (experiment 12, fig. 6). Briefly, if one Swingle unit or multiples thereof are incubated with constant amounts of hypertensinogen, the hypertensin yield is practically the same; this holds even when rather large quantities of hypertensinogen are available. It is difficult to reconcile the unchanged hypertensin yield in *in vitro* experiments with the well graded increment

in blood pressure response to injection of increasing amounts of renin into the living animal. Hessel (15) reported a direct relationship between the quantities

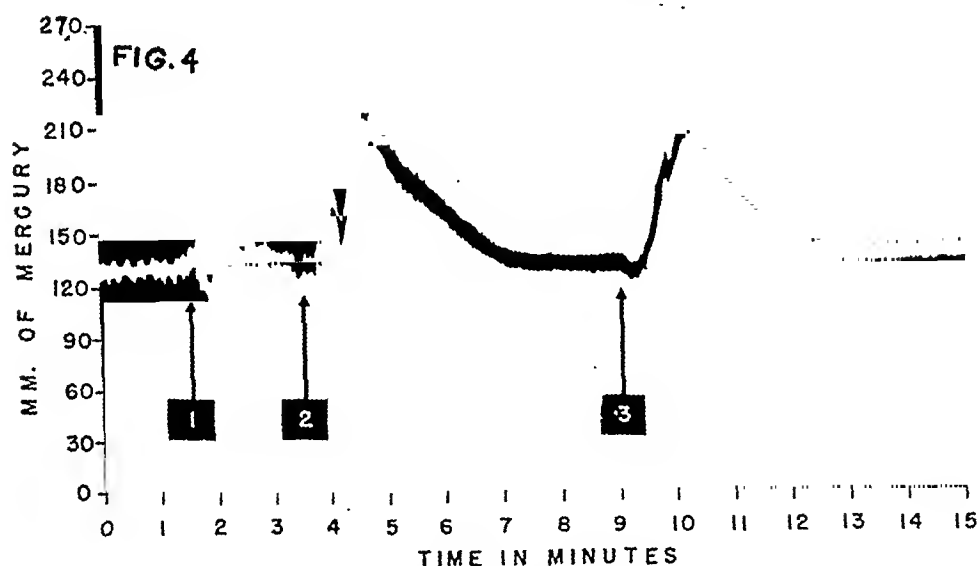
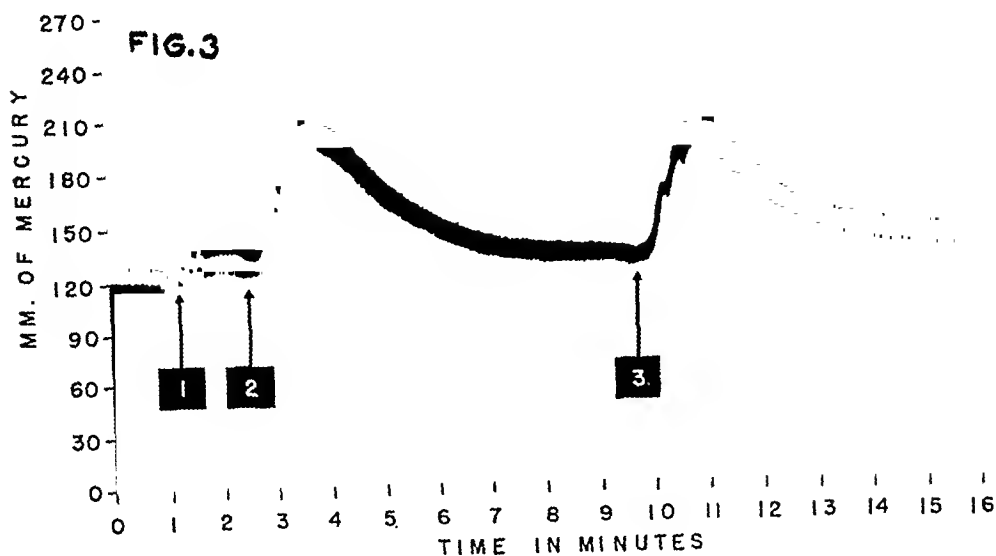


FIG. 3. BLOOD PRESSURE CURVES FOLLOWING I.V. INJECTION OF HYPERTENSIN produced by incubating 2.5 units of renin with 100 cc. of dog serum for: (1) control (no renin added to the serum), (2) one-half minute, (3) one and one-half minutes (Exp. 8, table 1).

FIG. 4. BLOOD PRESSURE CURVES FOLLOWING I.V. INJECTIONS OF HYPERTENSIN produced by incubating 2.5 units of renin with 100 cc. of dog serum for: (1) control (no renin added to the serum), (2) one-third minute, (3) three minutes (Exp. 9, table 1).

of intravenously injected renin and the blood pressure response, and suggested a blood pressure unit' *E*.

Other investigators reported similar results (16). Swingle and associates (12) defined a renin unit and reported a response of 32 to 52 mm. of Hg for one unit, 55 to 96 for two, 62 to 119 for three and 95 to 132 for four.

Discrepancies are evident between the action of medium amounts of renin (one to 3 Swingle units) *in vitro* and their pressor action *in vivo*. Definite differences are also demonstrable when the effects of the injection of large amounts of renin are compared with the effect of large amounts of hypertensin. Pressure

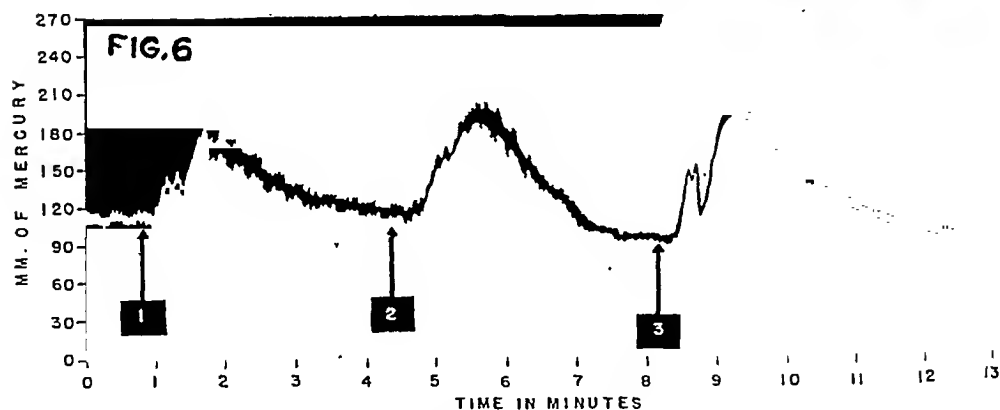
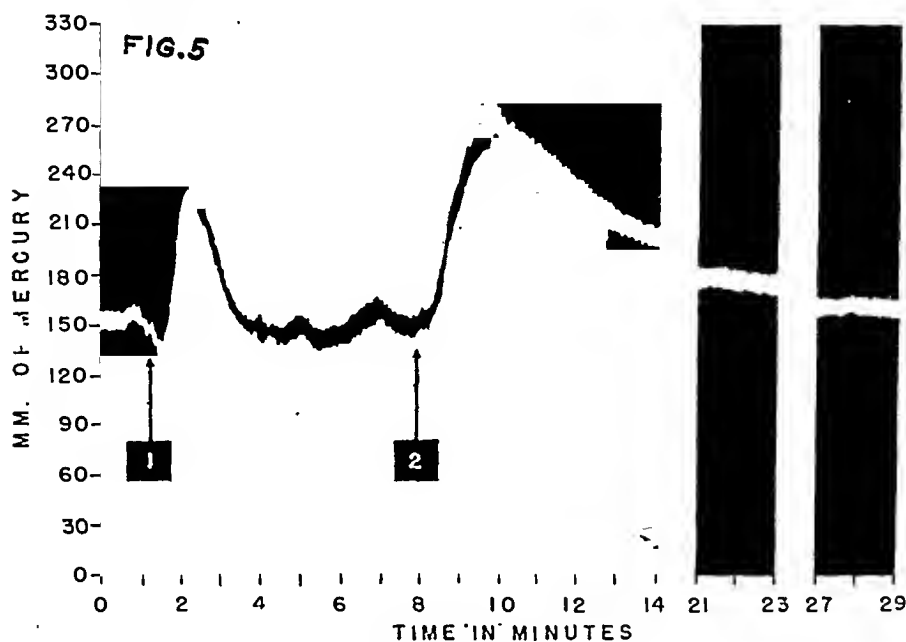


FIG. 5. BLOOD PRESSURE CURVES FOLLOWING: (1) i.v. injection of six units of hypertensin, (2) i.v. injection of four units of renin.

FIG. 6. BLOOD PRESSURE CURVES FOLLOWING I.V. INJECTIONS OF HYPERTENSIN produced by incubating different amounts of renin with constant amounts of hypertensinogen (100 cc. of dog serum). (1) one unit of renin used; (2) three units of renin used; (3) nine units of renin used (Exp. 12, table 1).

rises of 160 to 180 mm. of Hg were often observed following injections of 8 to 10 units of renin (16) and Hessel (15) even reported rises of 250 mm. and more. On the other hand, the maximal blood pressure elevations observed in these studies following injection of hypertensin did not exceed 100 mm. of Hg. These results are in agreement with those of Braun-Menendez and associates. The

latter authors observed maximal elevations of not more than 90 to 100 mm. of Hg, with up to 20 units of hypertensin (14).

Hypertensin yield with increasing quantities of hypertensinogen. The great amounts of hypertensin used in the experiments reported in this paper were prepared with rather large quantities of hypertensinogen. Table 2 shows the relation between hypertensin yield and availability of hypertensinogen.

The dependency of the hypertensin yield on the available hypertensinogen is evident and has been reported previously (14). The influence of the quantity of renin used in this series is insignificant, as can be seen from the similar hypertensin yield.

The reason for the different action of renin *in vivo* is obscure. Renin still is a crude mixture containing many enzymes. The possibility arises that it may contain a substance which acts directly on effector cells and that this second

TABLE 2. RELATION OF HYPERTENSIN YIELD TO THE AVAILABLE HYPERTENSINOGEN

EXPERIMENT	RENIN UNITS 'SWINGLE'	1% HYPER- TENSINOGEN	FRACTION OF THE TOTAL HYPERTENSIN YIELD INJECTED	BLOOD PRESSURE RISE	WEIGHT OF DOG
		cc.		mm. Hg	kgm.
13	4	40	Total	57	7
	4	80	One half	63	
	4	320	One fourth	66	
14	1	20	Total	42	7.2
	1	80	One half	65	
	1	160	One half	90	
15	2	20	Total	30	5.4
	2	100	One half	30	
	2	150	One fourth	40	
	2	310	One sixth	50	

effect may be demonstrable. An effort to accomplish this involved amputation and perfusion of one ear of a rabbit, intravenous injection of renin or hypertensin followed by removal and perfusion of the second ear.

Preliminary study showed that the initial perfusion rates of both ears of normal rabbits did not vary significantly when the ears were amputated within 30 minutes of each other. The variations with 10 pairs of ears were only 10 to 20 per cent of the drop rate.

The effect of intravenous injection of renin. One ear of a normal rabbit was amputated and the basal drop rate determined. Two to three 'Swingle' dog units of renin were then injected into the animal intravenously. Five to fifteen minutes later the second ear was amputated and perfused with 20 cc. of Ringer-Locke to insure absence of blood. The initial drop rate of the second ear in each of 12 perfusion experiments with Ringer-Locke was 40 per cent to 98 per cent less than that of the control ear and remained at this rate for at least five minutes. On continuation of the perfusion with Ringer-Locke it gradually returned over a period of 10 to 30 minutes toward the basal rate of the control ear.

The effect of intravenous hypertensin injection. The identical experiments were carried out in six rabbits using intravenous hypertensin injections instead of renin, with markedly contrasting results.

TABLE 3. RENIN INFLUENCES THE VESSELS OF THE RABBIT'S EAR IN A MANNER DIFFERENT FROM THAT OF HYPERTENSIN

EXPERIMENT	EAR	DROP RATE PER $\frac{1}{2}$ MIN.	RENIN UNITS 'SWINGLE'	TIME IN MINUTES FROM INJECTION TO AMPUTATION OF EAR
16	First	28	—	—
	Second	4	3.0	.5
17	First	38	—	—
	Second	1	2.5	5
18	First	28	—	—
	Second	5	3.0	5
19	First	26	—	—
	Second	10	2.0	5
20	First	44	—	—
	Second	5	3.0	6
21	First	38	—	—
	Second	14	2.0	5
			HYPERTENSIN UNITS	
22	First	23	—	—
	Second	53	3.0	1.5
23	First	24	—	—
	Second	30	3.0	1.25
24	First	34	—	—
	Second	50	3.0	1.0
25	First	18	—	—
	Second	30	6.0	0.75
26	First	20	—	—
	Second	55	6.0	0.75
27	First	30	—	—
	Second	46	7.0	0.75

After the basal drop rate of the first ear had been determined, 3 to 7 units of hypertensin were injected into a rabbit. At the peak of the pressor response (between 45 and 90 seconds after the injection) the second ear was removed, flushed out with 20 cc. of Ringer-Locke and the drop rate measured within three

minutes of the time of the hypertensin injection. In no instance was there any reduction in drop rate; there was rather a prompt increase, indicating vasodilation, which subsided after a minute or two to the basal level of the first ear. Table 3 contains the data of typical experiments.

SUMMARY AND DISCUSSION

Amounts of renin just detectable by bioassay in a dog are capable of rapidly and completely transforming large quantities of hypertensinogen. An increase in renin over and above these amounts does not augment the hypertensin yield significantly, the latter depending exclusively upon the available amounts of hypertensinogen. No evidence is at hand to indicate that the quantities of hypertensinogen in the blood can increase consequent to injection of renin. It may be concluded, therefore, that the proportional increase in blood pressure that follows the intravenous injection of increasing amounts of renin cannot be explained by a greater formation of hypertensin.

Differences between the renin and hypertensin effect on blood vessels have been demonstrated: A constrictor effect of intravenously injected renin on the vessel walls of the rabbit's ear is observable after the organ has been removed from the body and perfused with Ringer-Locke for many minutes. No such effect can be demonstrated following an injection of hypertensin.

The possibility is suggested that an unknown factor contained in renin may either prolong or intensify the action of hypertensin or may act independently of it. Recent reports are of interest in this connection. Tripp (17) suggests that hypertensin (angiotonin) lacks some pressor material resulting from the renin and substrate reaction. Helmer and Shipley (18) believe they have evidence of the presence of a pressor substance distinct from renin and originating in the kidney.

Results included in this communication permit the conclusion that the *in vivo* renin effect is not adequately explained by hypertensin formation.

We acknowledge the valuable technical assistance given by Miss Helen Criscuolo, Mr. Edward Iannucci and Mr. Peter Integlia.

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EFFECT OF INTRAVENOUS INJECTION OF MAGNESIUM CHLORIDE ON THE BODY TEMPERATURE OF THE UNANESTHETIZED DOG, WITH SOME OBSERVATIONS ON MAGNESIUM LEVELS AND BODY TEMPERATURE IN MAN

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Received for publication December 5, 1947

The mean serum magnesium concentration in normal persons has been reported variously as from 2.00 to 2.74 mgm. per cent, with a range from 1.60 to 3.66 mgm. per cent (5). Haury and Cantarow (5) found in 52 subjects normal limits from 1.7 to 3.1, with a mean of 2.48 mgm. per cent. Higher concentrations produce well known pharmacological actions (reviewed by Smith, Winkler and Hoff, 12). Vasodilation occurs with concentrations as low as 2 to 5 mEq. per liter (1 mEq. equals 1.2 mgm. per cent), and this is, according to Haury (4), a peripheral action, and according to Winkler *et al.* (17) an action upon the arterioles. Greater concentrations affect the nervous system, first with a 'curare-like' action of retarding neuromuscular transmission (7, 16). With serum concentrations of 6 to 14 mEq. per liter muscles fail to respond to single shocks through the nerve, and with higher concentrations the response to first low and then high frequency stimulation disappears. Still higher concentrations cause depression of the central nervous system.

The temperature-depressing effects of magnesium are less well known, though described in 1916 by Schütz (11) in rabbits. He thought the action was on the temperature-regulating centre, but this is not certain from his work in view of the known peripheral actions of the drug. A number of papers from the laboratory of H. G. Barbour (17, 18) also fail to give conclusive evidence as to the site of action and mechanism. Our interest was renewed on reading that Suomalainen (13, 14) had found an elevated ratio of magnesium to calcium in the serum of hibernating hedgehogs. A single experiment by one of us in 1937 on an unanaesthetised cat gave the result that magnesium injection caused panting at a lower body temperature than usual and suppressed shivering on cooling.

Since magnesium, unlike most antipyretics, is a simple inorganic ion and is somehow concerned in hibernation, a specific effect on the temperature-regulating centre, if it could be demonstrated, would be of considerable interest and might even suggest that the level of serum magnesium was concerned in the normal regulation of body temperature.

Since the centre is extremely sensitive to anaesthetics (6), studies on the normal regulation must be made on unanaesthetised animals, and dogs were chosen for this reason. The plan was to study the mechanism of lowering of body temperature after injection of magnesium in three sets of conditions; a) at

¹ This work was carried out with the aid of a Medical Research Fellowship from the National Research Council, Canada.

ordinary room temperature, where there was initially neither shivering nor panting; b) while the animal was being warmed and panting moderately; and c) while the animal was cooled and shivering moderately. In each case the particular regulating mechanism involved could show increase, decrease or no change.

METHOD

For each experiment the dog was placed on the animal table and wrapped in a special rubber 'blanket' through which warm or cold water could be circulated under controlled conditions to warm or cool the animal (fig. 1). The deep rectal temperature and the surface temperature of the large pad of the right forepaw were taken by means of thermocouples. The paw temperature served

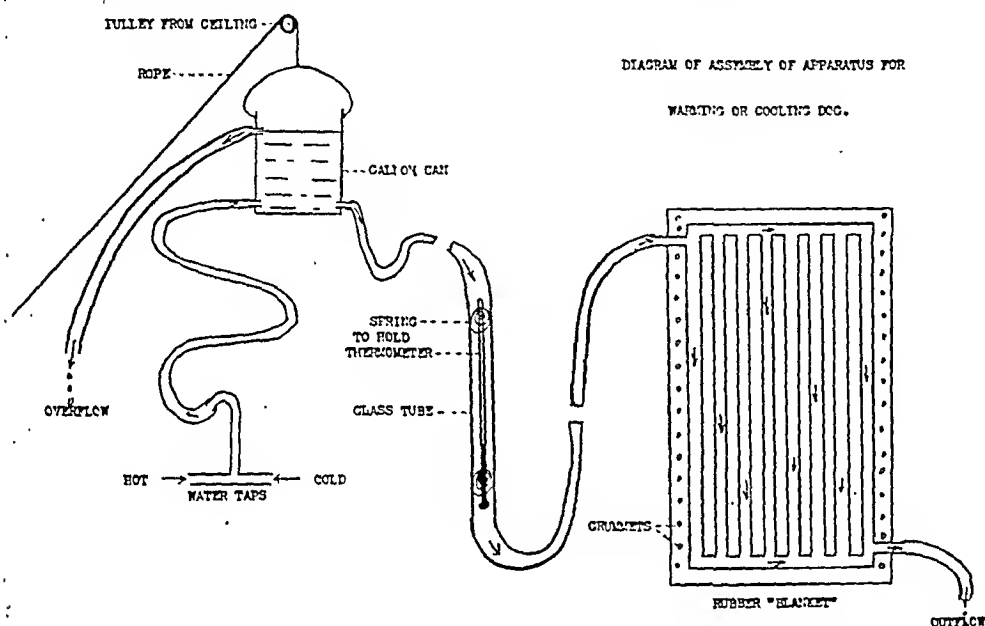


FIG. 1. DIAGRAM OF ASSEMBLY OF APPARATUS for warming or cooling dog.

as an indication of vasoconstriction or vasodilation. When the rectal and paw temperatures had become stabilized molar magnesium chloride in various amounts was injected into a saphenous vein and the effects observed.

Samples of venous blood were withdrawn during the experiments and the serum magnesium concentrations were estimated either by a modification of Hoffman's method (8) using 8-hydroxyquinoline, or, in later experiments, by a modification of Garner's method (3) using Titan yellow.

The degree of shivering or of panting, when present, was estimated by inspection, and for comparative purposes was evaluated as 1, 2, 3 or 4 plus. In the warming experiments the apparatus was adjusted so that before the injection the dog maintained a stable rectal temperature by moderate intermittent panting, and this was recorded as two plus panting. In cooling experiments the temperature of the cooling water (about 7°C) was such that initially the dog

maintained a stable rectal temperature by means of vasoconstriction and moderate shivering. This is recorded as two plus shivering.

OBSERVATIONS

General effects of injection of magnesium. Usually the dog had been quite active before entering the apparatus and was warm and panting at the beginning of the experiment. The animal became quiet in the apparatus and the paw and rectal temperatures became stabilized. When magnesium was injected intravenously the effect varied with the dosage, from mild or moderate depression

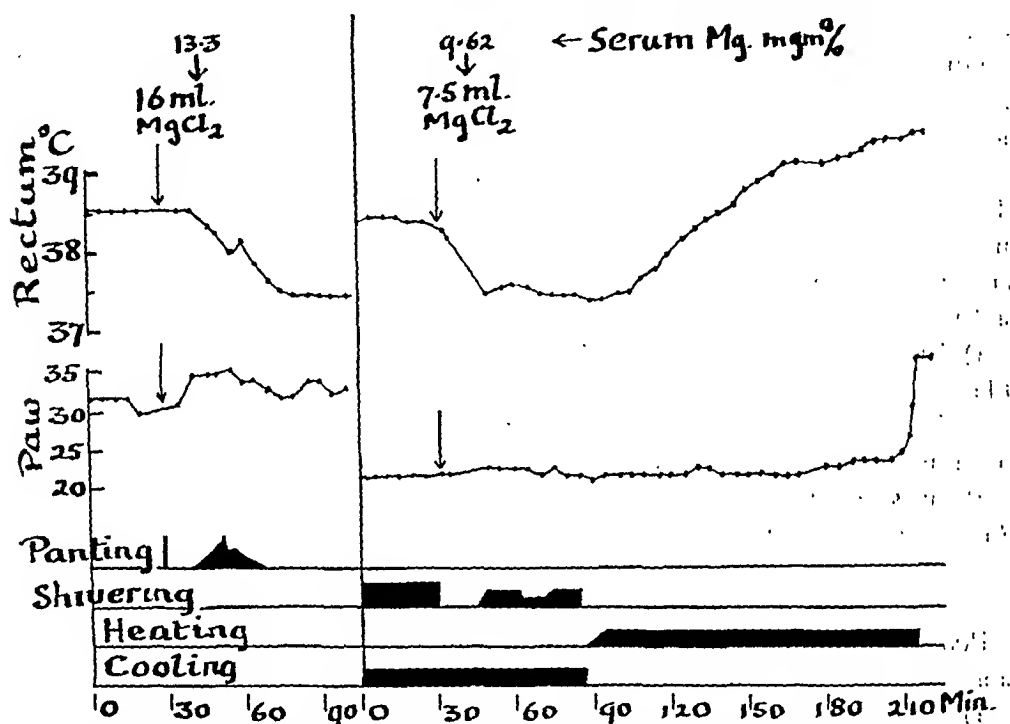


FIG. 2. CHARTS OF TWO EXPERIMENTS. Left: 20-kgm. dog at room temperature. Right: 10-kgm. dog first cooled, then warmed. Molar magnesium chloride was injected intravenously at the point shown by the arrows.

to complete paralysis or even narcosis. In all the experiments the dog salivated profusely during and immediately following the injection. In almost all the experiments there was a short period of retching after the injection. The dog usually became drowsy during part of the time after the injection, and there was depression of the Achilles' tendon reflex, which was from moderate to complete, depending on the dose.

Effects on temperature regulation. Figure 2 shows two experiments, the first at room temperature and the second with cooling followed by warming, to illustrate the results. The drop of rectal temperature after injection of magnesium in each case indicates the antipyretic action, but the mechanism is obviously different. In the first experiment, the rise of paw temperature indicates a

vasodilation and there was also stimulation of panting. In the second case, during cooling, shivering was inhibited, while there was no evidence of panting or vasodilation, even though the latter occurred reflexly after warming (at time 210 minutes). The results of 20 experiments on three dogs are summarized in table 1, where only the maximal changes of rectal and paw temperatures are given. The mean values at the foot of the tables should of course be regarded with suspicion, as the data is heterogeneous, but they serve to summarize the findings.

When panting was present it was typical polypneic panting with the mouth held open, rapid rhythmic shallow respiration, and rhythmic movements of the tongue and labial commissures synchronous with respiration. When the dose of magnesium was high enough to produce symptoms of paralysis at the same time that panting was stimulated, there was a striking contrast between the efforts of the animal to pant and the general depression.

Injection of magnesium was always followed by a fall of rectal temperature. However, the changes occurring in peripheral blood flow (as shown by changes in paw temperature), in panting or in shivering when present, differed according to the condition of the animal with regard to temperature regulation at the time of the injection. From table 1 the effects can be summarized as follows:

a) In an animal being warmed there was an increase in panting, and a vasodilation in the cases where this was not already maximal.

b) In an animal neither warmed nor cooled there was vasodilation always, often accompanied by panting.

c) In an animal being cooled there was a decrease or cessation of shivering. Vasoconstriction, however, persisted.

DISCUSSION

Possible mechanisms by which these changes in temperature regulating functions could be produced are;

a) Mechanisms independent of the temperature-regulating centre, such as 1. peripheral vasodilation 2. paralysis of the neuromuscular junction by a curare-like action of magnesium and 3. a depression of metabolism.

b) Mechanisms involving the centre, such as 1. depression of temperature regulation against cold, either specific or part of a general depression of the central nervous system, and 2. stimulation of the central regulation against heat.

The one feature that is common to all the experiments, in the different conditions, is a fall of rectal temperature after the injection of magnesium chloride. Since control injection of sodium chloride did not affect body temperature (and this is well known), it is concluded that the effect is due to the magnesium ion.

Because of the pharmacological actions of magnesium it is necessary to consider certain possibilities whereby the body temperature might be affected independently of the temperature-regulating centres. Magnesium is a powerful vasodilator, and this action is reputed to be peripheral (4, 17). Also, magnesium exerts a powerful paralytic action at the neuromuscular junction—its so-called 'curare-like' action (7, 16). Either of these actions could influence temperature

TABLE 1.—SUMMARY OF EXPERIMENTS

WT. IN KILOS	ROOM TEMP.	M. MgCl ₂ INJECTED	CHANGE IN RECTAL TEMP. AFTER Mg	PAW TEMP.		PANTING		REMARKS
				Initial temp.	Change on injtn.	Before	After	
A. Dog neither warmed nor cooled								
9.6	21	4.8	-0.40	Stayed warm		0	++++	Rectal temp. still falling when expt. ended. Paw temp. estimated by palpation only.
20	24	16.0	-1.04	29.7	+5.4	0	++++	Panted during injtn., evidence of narcosis by Mg. Increased panting when narcosis wore off.
20	26	5.0	-0.35	34.2	+0.9	0	+++	Serum Mg 4.23 mg. % 25 mins. after injection.
10	25	5.0	-0.35	34.4	+1.2	0	++	Serum Mg 9.04 mg. % 22 mins. after injection.
10	22	7.5	-0.98	22.4	+9.6	0	0	Serum Mg 10.04 mgm. % 7 mins. after injection.
10	24	10.0	-0.95	22.5	+4.3	0	++++	Serum Mg 10.9 mgm. % 9 mins. after injection.
10	26	9.5	-0.49	33.0	+1.4	Occas. panting	+++	Serum Mg 2.04 mgm. % before, 7.60 mgm. % 12 mins. after injection.
Mean values...	24		-0.65	29.4	+3.8	0	+++	
B. Dog warmed								
9.6	27	4.8	-0.87	36.7	+1.5	++	++++	First expt. on this dog. Struggle during injtn. raised rectal temp. Constriction may be psychogenic.
20	21	5.0	0	36.1	-3.8	++	++++	
9.6	26	9.6	-1.43	36.0	+0	++	++++	Curare-like action of Mg obvious. Panting very severe.
20	20	6.0	-0.56	25.5	+9.0	++	++	

TABLE 1—CONTINUED

WT. IN KILOS	ROOM TEMP.	M. MgCl ₂ INJECTED	CHANGE IN RECTAL TEMP. AFTER Mg	PAW TEMP.		PANTING		REMARKS
				Initial temp.	Change on injn.	Before	After	
10	°C. 27	ml. 5.0	°C. -0.25	35.3	+1.2	+	++++	Serum Mg 2.33 mgm. % before, 7.12 mgm. % 16 mins. after injtn.
10	27	7.5	-0.25	34.9	+1.2	0	++	Serum Mg 9.87 mgm. % 12 mins. after injection.
10	24	7.5	-0.32	33.5	+0.5	++	++++	Serum Mg 2.23 mgm. % before, 7.59 mgm. % 15 mins. after injection.
Mean values...	25		-0.61	33.7	+2.2	+or++	++++	2nd expt. omitted from mean.

C. Dog cooled

						SHIVERING		
						Before	After	
9.6	16	4.8	-0.50	17.5	-1.4	++++	++	Some struggling on injection.
20	19	1.0	-0.04	23.3	-0.3	+++	++	
20	23	10.0	-0.88	26.3	+2.7	++	0	Panting ++++ immediately on injection. Serum Mg 6.0 mgm. % 30 mins. after injection.
10	21	5.0	-1.05	22.0	+1.0	++	0	Rectal temp. still falling at end of expt. Serum Mg 2.73 mgm. % before, 6.44 mgm. % 10 mins. after injection.
10	23	7.5	-0.90	21.4	+1.1	+++	0	Serum Mg 9.62 mgm. % 15 mins. after injection.
20	20	15.0	-0.81	21.1	+3.2	++	0	
Mean values..	20		-0.70	21.9	+1.0	+++	+	

without directly affecting the temperature-regulating centres. Increased concentrations of magnesium cause central depression and narcosis, and it is possible that general depression would also depress temperature regulation. The prob-

lem is to distinguish any action on the temperature-regulating centres from these other nonspecific actions of the ion.

Vasodilator action. In the warming experiments and most experiments at room temperature, marked vasodilation occurred if it was not present initially. This is in accord with the accepted vasodilator action of magnesium. However, in the cooling experiments vasoconstriction was maintained despite the reported peripheral vasodilator action of magnesium. It appears, therefore, that the vasoconstrictor centre is not significantly depressed by magnesium in the amounts used, for it overrides the tendency of magnesium to cause peripheral vasodilation.

Action on shivering—neuromuscular paralysis or central depression? In the cooling experiments magnesium consistently caused a decrease or cessation of shivering without marked evidence of vasodilation. The effect on shivering could be due to depression of the centre responsible for shivering. If so, the depression might be part of general depression of the central nervous system, or

TABLE 2.—MEAN SERUM MAGNESIUM CONCENTRATIONS OF SUBJECTS ARRANGED ACCORDING TO ORAL TEMPERATURE

ORAL TEMPERATURE	MEAN SERUM MAGNESIUM CONCENTRATION MGM. PER 100 CC.	
	Healthy subjects	Patients
°F.		
97.0-97.4	2.25 (7) ¹	
97.5-97.9	2.28 (10)	2.29 (2)
98.0-98.4	2.22 (21)	2.29 (7)
98.5-98.9	2.36 (7)	2.25 (7)
99.0-99.4		2.29 (2)
99.5-99.9		2.23 (1)

¹ Figures in brackets show number of observations.

it might be specific on the temperature-regulating centre. In some experiments the dog showed gross paralysis, and in these at least there is no doubt that a curare-like effect of the ion could be adequate to explain the effect on shivering. In other experiments gross paralysis did not occur, but there were usually signs of depression, and in general there was a positive correlation between the fall in rectal temperature and the evidence of general depression. This is in agreement with Schütz (11), who noted a relation between the temperature depression and the symptoms of paralysis after administration of magnesium to rabbits. It is possible, therefore, that the curare-like action of magnesium could account for the effects on shivering. There may be also a central effect that influenced shivering, but it was not distinguished in these experiments from general depression of the central nervous system.

Action on panting—stimulation or depression? We have seen that temperature depression by means of vasodilation, and by means of decrease or cessation of shivering, might be explained on the basis of accepted peripheral actions of the magnesium ion. The effect on panting, however, can not be explained by known peripheral actions of the ion. By its nature, panting must be a central effect.

Superficially, it would appear to represent stimulation of regulation against heat. But Lilienthal and Otenasek (9) have described a panting centre that is separate from the hypothalamic centres that regulate body temperature, and they point out the possibility of release phenomena affecting panting. It is possible, therefore, that magnesium may cause panting by releasing such a centre from controlling influences, and if this were so it would be consistent with the general depressing action of magnesium (10). However, against this is the observation that during general depression by means of nembutal, used in one warming experiment, panting stopped and body temperature rose. In any case, panting is increased by magnesium injection, and any argument as to 'direct' stimulation of a centre or 'release' from the influence of other centres by their depression is largely academic, especially in view of the well-known reciprocal actions in the central nervous system.

One possible mechanism mentioned for the effect of magnesium on body temperature is depression of metabolism. In the present series of experiments no attempt has been made to investigate an effect of magnesium on resting metabolism, because the obvious effects of magnesium—panting, decrease in shivering and increase in paw temperature—are of such importance to temperature regulation that the first problem is to determine whether they represent a true antipyretic effect, mediated through the temperature-regulating centres. Berta and Györi (2) found that subcutaneous injection of magnesium gluconate lowered the oxygen consumption of the rat 20 to 40%. It might be supposed that this effect was secondary to neuromuscular paralysis by magnesium, but these authors point out that Tangl and Verzar (15) found that in animals that were accustomed to metabolism experiments neuromuscular paralysis by curare did not lower the metabolic rate.

CORRELATION OF SERUM MAGNESIUM LEVELS AND BODY TEMPERATURE IN MAN

To investigate the possibility that magnesium may play a physiological role in determining the normal body temperature, clinical observations were made of the serum magnesium concentration in relation to the body temperature at various times of day. Sets of two or more observations in a 24-hour period were made on 10 normal subjects and six ambulatory patients. Forty-five determinations on 10 healthy subjects gave a mean value of 2.26 ± 0.33 SD mgm. per cent and 19 on ambulatory patients gave 2.26 ± 0.15 SD, the total 64 determinations yielding a mean of 2.26 ± 0.30 SD. The results are listed in relation to the levels of oral temperature in table 2, and it is apparent that there is no clear correlation. This was further tested by comparing the magnesium levels for each subject when the lowest oral temperature and the highest oral temperature were recorded. The results are shown in table 3. Again, it is seen that there is no constant trend.

An additional series of observations was made on six patients receiving artificial fever therapy in the fever cabinet and on one patient who had artificial fever induced by typhoid vaccine. In the patients in the fever cabinet the mean serum magnesium concentration was 2.32 ± 0.30 SD mgm. per cent before fever

when the mean temperature was 99.2°F, and 2.19 ± 0.26 SD mgm. per cent at the height of fever when the mean oral temperature was 105.2°F. The patient who received vaccine did not develop much fever, and successive temperature and magnesium figures were 98.6° and 2.45 mgm. per cent; 99.6° and 2.40 mgm. per cent; and 100.6° and 2.39 mgm. per cent. It is apparent that a large number of cases would be needed to prove a significant relation, but in any event the change in magnesium concentration with change of body temperature, in the physiological range, is too small to lead to significant physiological effects.

SUMMARY

1. On intravenous injection into the dog magnesium chloride causes a decrease in rectal temperature whether the dog is in an environment that is warm, cool or at normal room temperature.

TABLE 3.—COMPARISON OF MEAN SERUM MAGNESIUM CONCENTRATIONS WHEN THE LOWEST ORAL TEMPERATURES WERE RECORDED WITH THOSE WHEN THE HIGHEST ORAL TEMPERATURES WERE RECORDED

	MEAN TEMPERATURE	MEAN SERUM MAGNESIUM CONCENTRATION
	°F.	mgm. per 100 cc.
18 sets of observations on 10 normal subjects	Low..... 97.7	2.16
	High..... 98.3	2.32
6 sets of observations on 6 patients	Low..... 98.2	2.38
	High..... 98.9	2.23
24 sets of observations on 16 subjects (Total)	Low..... 97.8	2.22
	High..... 98.4	2.29

2. In a cold environment the effect is produced by a decrease of shivering and of voluntary motor activity without vasodilation.

3. In a comfortable environment the effect is produced either by panting or by vasodilation or a combination of these, and probably by a decrease in muscular tone in some cases.

4. In a warm environment the effect is produced by an increase in panting.

5. It is probable that the decrease in shivering is due to the curare-like action of magnesium on the neuromuscular junction. If there is depression of the temperature-regulating centre, it has not been distinguished from the general depression of the nervous system.

6. The experimental results are not inconsistent with the view that the vasodilator action of magnesium is a peripheral action. However, in the shivering animal the vasoconstrictor centre overrides the vasodilator action of magnesium.

7. The increase in panting must result from a central effect of magnesium. While this appears to represent stimulation of the panting centre, it may of course be due to a 'release phenomenon' when other 'inhibitory' centres are depressed.

8. There is no clear evidence that magnesium exerts an antipyretic action spe-

cifically through the temperature-regulating centres. However, its combined actions change the level of *effective* regulation in favour of a lower body temperature rather than merely depressing the temperature-regulating mechanism.

9. In a short series of investigations on healthy human subjects and ambulatory patients no evidence was found that there is a significant relation between the diurnal fluctuation of normal body temperature and the serum magnesium concentration. Neither was a significant relation found between the serum magnesium concentration and the body temperature during artificial fever. The changes in magnesium level with body temperature are too small to be of physiological importance.

We wish to express our thanks to Mrs. Martha McIntosh for her able assistance in conducting the experiments reported in this paper.

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EFFECT OF PULMONARY ARTERY LIGATION ON THE HISTAMINE CONTENT OF LUNG, WITH OBSERVATIONS ON CONCOMITANT STRUCTURAL CHANGES¹

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Received for publication November 5, 1947

The high concentration of histamine in the lung, and in certain species the liver, suggests that those organs which first receive the venous effluent from the various tissues may function to remove histamine from the systemic circulation and store it in an inactive form (1). To test this hypothesis the effect of pulmonary artery ligation over varying periods of time on the histamine content of the dog lung was determined. It was reasoned that if pulmonary tissue functions to filter and store histamine, marked differences in histamine concentration between ligated and control lobes would become apparent over a period of time.

Early experiments were of short duration in order to determine whether or not ligation of the pulmonary artery would be compatible with the maintenance of structural integrity. The ability of the bronchial circulation to maintain the pulmonary parenchyma, a finding which will be discussed in more detail in a later paper, permitted long range observations which afforded a definitive answer to the effect of pulmonary artery ligation on the histamine content of lung tissue.

METHODS

Ten stock dogs weighing around 10 to 15 kilograms were used. Anesthesia was induced with pentobarbital and supplemented when necessary by the intratracheal insufflation of ether-air mixtures through a soft rubber catheter. Under aseptic technic, an intercostal incision was made in the right chest. The right middle lobe was removed by individual dissection and ligation of pulmonary artery, vein and bronchus in that order and preserved for histamine assay. The pulmonary artery branch to the lower lobe was then freed and ligated doubly with silk in continuity. The remaining lobes were protected against direct trauma during the course of the operation and carefully reinflated to fill the pleural cavity during the closure. There were no operative deaths during the series.

At intervals ranging from two weeks to eight months, the animals were killed by overdosage with intravenous pentobarbital. At autopsy the condition of the remaining lobes in situ was noted. The occlusion of the pulmonary artery to right lower lobe was verified. The right and left lungs were removed separately. In the later animals of the series the weight and collapsed volume of the right and left lower lobes were recorded. The bronchi of the two lobes were then cannulated, and the two lobes brought simultaneously to inflation by air injection

¹ Aided by a Grant from the Fluid Research Funds, Yale University School of Medicine.

under control with a water manometer. The cannulae were then occluded and the expanded volume of each lobe measured by water displacement; the average of three determinations was recorded. In all experiments representative specimens from the peripheral and hilar portions of the middle lobe (excised at operation), and the lower lobes (removed at autopsy) were weighed and placed in a refrigerator until treated within a few hours according to the procedure outline under assay technic. Additional specimens were immersed in ten per cent formalin and prepared for histologic study by hematoxylin-eosin and Masson connective tissue stains.

Assay technic. Tissues were kept in the refrigerator from the time they were obtained until extracted, usually a period of no more than six to eight hours. Control experiments showed that pulmonary tissue may be kept at icebox temperature for 48 hours without any change in histamine content (2).

From one to three grams of tissue was extracted for histamine assay. The samples were thoroughly macerated by grinding with sand, the tissue pulp and sand were transferred to a wide-mouthed bottle and extracted for three hours in a mechanical shaker with 50 cc. of 70 per cent alcohol containing sufficient HCl to make a 0.1 N solution. The supernatant fluid was decanted, the residuum re-extracted, and the combined extracts were centrifuged. The resultant supernatant fluid was evaporated to dryness on a steam bath. The extract was kept in the form of this dried residue until the time of bioassay. Immediately prior to bioassay, the dried residue was taken up in an amount of Tyrode's solution such that the extractives of each gram of the original lung sample were dissolved in 5 cc. The resultant solution, free of sediment, was employed in the bioassay and either used as such or further diluted with Tyrode's solution.

Assay was performed on isolated segments of guinea pig ileum suspended in 3 cc. of oxygenated Tyrode's solution containing one mgm./liter of atropine sulfate. Samples from the previously resected right middle lobe, the right lower lobe and the left lower lobe were assayed on the same strip of ileum using crystalline histamine as the standard.

The technic of extraction described above fulfills the known requirements for the quantitative extraction of histamine from tissue and is simple in execution. Seventy per cent acid alcohol not only supplies a medium in which histamine is readily soluble but also acts as a bactericidal agent and a protein precipitant. Thus the formation of histamine by bacterial action is circumvented and the bound cellular histamine is released. Control experiments have shown that 100 per cent of known amounts of histamine added to tissue can be recovered by this procedure.

RESULTS

The results of these experiments are presented in two categories: first, the effect of excluding the pulmonary arterial flow on the histamine content of the right lower lobe as compared with the content in the preoperative (R.M.L.) and postoperative (L.L.L.) controls; secondly, the observed effect of pulmonary artery ligation on the structural integrity of the lobe. While the latter was not a pri-

mary purpose of the experiment, the results are of considerable interest and appear worthy of recording.

The data of table 1 show that with a single exception the long-standing exclusion of mixed venous (pulmonary artery) blood from a single lobe for a period ranging from 15 to 256 days had no appreciable effect on the tissue histamine level as determined by the bioassay method.

TABLE 1. HISTAMINE LEVELS IN PULMONARY TISSUE
(Micrograms per gram, wet weight)

	DOG NUMBER									
	15	16	17	18	19	20	21	24	25	26
Duration ligation in days..	56	29	15	86	168	196	205	256	226	220
Right middle lobe (pre-operative control).....	60	55	8	77	24	35	12	17	17	13
Right lower lobe (ligated artery).....	66	49	11	66	9	33	12	25	29	13
Left lower lobe (postoperative control).....	60	30	12	66	11	33	12	20	41	14

TABLE 2. VOLUME AND WEIGHT RELATIONSHIPS

	DOG NUMBER						
	18	19	20	21	24	25	26
Duration (days).....	86	168	196	205	256	226	220
Volume collapsed (cc.)							
Right lower lobe.....	—	—	—	—	35.5	44.5	45
Left lower lobe.....	—	—	—	—	35.0	45.0	47
Volume expanded (cc.)							
Right lower lobe.....	—	—	—	150	175	—	205
Left lower lobe.....	—	—	—	200	235	—	335
Inflation pressure (cm. H ₂ O).....	—	—	—	15	26	—	26
Weight (grams)							
Right lower lobe.....	15.3	25	20	27	18.3	35.5	22.0
Left lower lobe.....	16.4	20	20	28	17.9	35.5	22.3

Dashes indicate data not collected for individual experiment.

Table 2 summarizes the comparative weight, collapsed volume and expanded volume in certain later experiments, where these observations were collected. In the earlier experiments they were not done since interest had not yet centered on this phase of the problem.

In every instance at autopsy, the right lower lobe with ligated pulmonary artery was paler in color, slightly denser in texture and somewhat more resistant to inflation. The relative weights and *collapsed* volumes were, however, within expected limits of normal.

Adhesions between visceral and parietal pleura, and between lobes on the side of operation, were variable in extent, nearly absent in some specimens, and dense

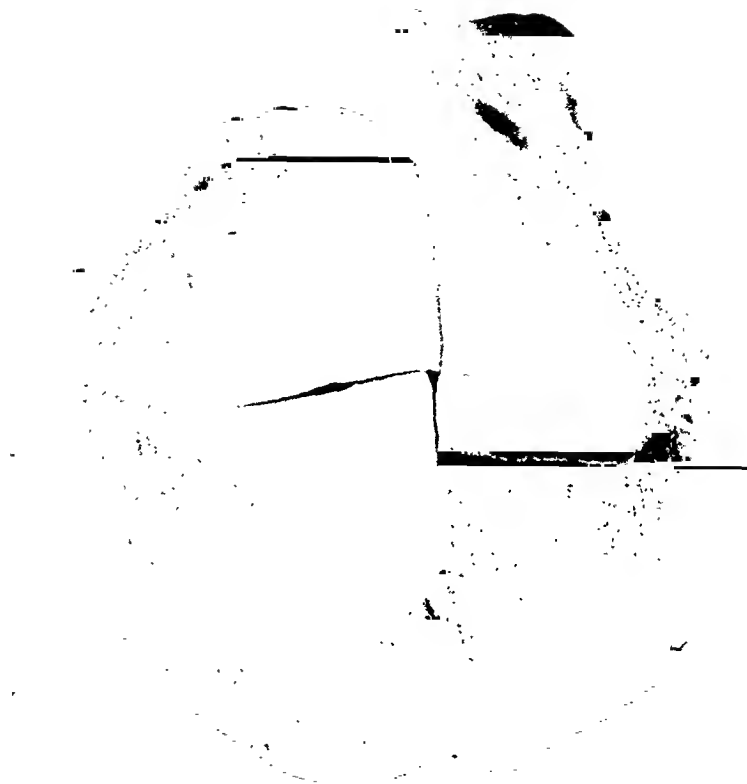


FIG. 1. PHOTOGRAPH OF EXCISED INFLATED LUNGS, dog 21. Note relative decrease in degree of inflation in right lower lobe and subpleural capillary enlargement adjacent to the fissure.

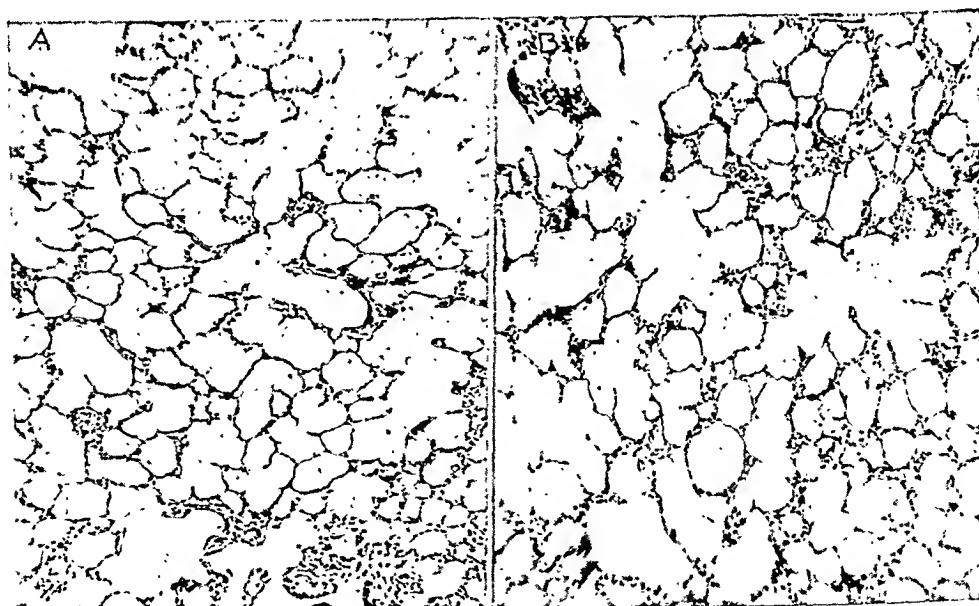


FIG. 2. A. PHOTOMICROGRAPH: right lower lobe section. *H* and *E* stain, 205 days after ligation of pulmonary artery to the lobe. Dog 21.
B. CONTROL SECTION from left lower lobe, same subject.

along the scars of the thoracotomy wound in others. Several specimens showed significant dilatation of the bronchial arterial branches and of the subpleural vessels (fig. 1), suggesting collateral channels.

Histologically, the architecture of the lobe deprived of pulmonary arterial circulation was well preserved (fig. 2a). The capillaries of the alveolar septa were reduced in size, and fewer red cells were in evidence, although some were present. Connective tissue stains showed a slight increase in fibrous tissue in the interlobular septa on the side of ligation.

There was no evidence at autopsy of atelectasis in any lobes, and no instance of gangrene.

DISCUSSION

The failure of pulmonary histamine levels to be significantly altered by long-standing deprivation of pulmonary blood flow renders unlikely the hypothesis that the maintenance of high histamine levels in the lung is dependent on a filtering or clearing function exerted by the lung parenchyma on the venous blood of the pulmonary artery. In other words, the 'storage' theory is given no support by these findings.

The stability of pulmonary histamine levels after pulmonary artery ligation is also in agreement with failure to demonstrate changes in vivo under other circumstances in which acute or chronic injury is done the *living* lung. The present authors have previously demonstrated (3) that experimentally induced lobar pneumonia in dogs does not alter the histamine levels if account is taken of the change in lung weight during consolidation. In some as yet unpublished data on the effect of a war gas (phosgene) a similar conclusion was reached. These observations support the hypothesis that histamine is firmly bound at some site in the lung tissues and is not readily released, short of actual tissue destruction.

The preservation of a nearly normal architecture and histology for many months after interruption of the pulmonary artery supply to a lobe is in agreement with the known function of the bronchial circulation in the nutrition of the pulmonary parenchyma. The persistence of a normal lobar weight and normal collapsed lobar volume demonstrates an absence of significant atrophy or atelectasis, while the reduction in expanded lobar volume after pulmonary artery ligation (as compared with the contralateral normal lobe) points to a loss in elasticity. This results from an increase in collagen in the interlobular tissues, which has been demonstrated by connective tissue stains.

SUMMARY

Operative interruption of the pulmonary arterial flow to the right lower lobes in ten dogs for periods ranging from two weeks to eight months produced no significant change in the levels of extractable pulmonary tissue histamine, as compared with preoperative and postoperative controls from the same animals. These findings suggest that the high concentration of extractable histamine nor-

mally present in the lungs is not the result of a pulmonary filtration, fixation and storage mechanism.

The structural integrity of the lobes deprived of pulmonary arterial blood supply has incidentally been studied. The weight and collapsed volume remain normal, while the expanded volume is diminished.

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BRAIN AND MUSCLE POTASSIUM IN RELATION TO STRESSFUL ACTIVITIES AND ADRENAL CORTEX FUNCTION¹

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Received for publication October 27, 1947

Pincus and Hoagland (1) and Pincus (2) have reported that a wide variety of stresses such as flying airplanes, the taking of examinations, exposures to heat or to cold and factory work result in enhanced output of adrenal cortical hormones. In addition, it appeared that one's fatigability, among other things, is related to the responsiveness of the adrenal cortex to stress.

The administration of the nontoxic, synthetic steroid $\Delta 5$ -pregnenolone to a group of pilots resulted in a reduced output of urinary 17-ketosteroids per increment of flight stress (3), and since low increments in excretion under stress had been found to correlate with greater resistance to fatigue, we found this of special interest. A number of studies were carried out by Pincus and Hoagland (3) by using subjects operating a pursuit meter when taking placebos and when taking $\Delta 5$ -pregnenolone, and in addition, investigations were made of the daily piecework production of three groups, comprising a total of 281 factory workers (4, 5). Significant improvement in performance of fatiguing psychomotor tasks was found when the workers were taking 50 to 100 mgm. per day of $\Delta 5$ -pregnenolone in contrast to placebos. This improvement in fatiguing psychomotor performance only occurred in situations which were really stressful and in which the subjects were well motivated.

The mechanism of the antifatigue action of pregnenolone is of interest. Adrenal steroids with an oxygen in the number 11 position are known to be primarily concerned with gluconeogenesis and in connection with this produce a fall in circulating lymphocytes (6). Steroids of the type of desoxycorticosterone (lacking an oxygen in the number 11 position) are primarily concerned with the regulation of salt and water metabolism (4, 10, 11, 30). $\Delta 5$ -Pregnenolone (hereafter referred to as pregnenolone) structurally resembles desoxycorticosterone more than it does other known adrenal steroids, since it also lacks an oxygen at its number 11 carbon atom.² We have, moreover, found that its

¹ This work was performed under Contract N6ori-197 of the Office of Naval Research. It was also aided by a grant from the Schering Corporation.

² Selye (see 26 for citations) found pregnenolone to have no depressive action on the pituitary. Pregnenolone differs from desoxycorticosterone in that its number 21 carbon atom is in the form of a methyl group instead of an alcohol group and it contains an hydroxyl group instead of an oxygen atom on the number 3 carbon. It also has a double bond between carbons 5 and 6 instead of between carbons 4 and 5, as in the case of desoxycorticosterone.

injection into rats does not alter the circulating lymphocyte count, nor does its ingestion in doses of 100 mgm. per day by mouth modify sugar tolerance curves in man (unpublished). It thus seemed to us likely that pregnenolone might play a role in regulating sodium and potassium in tissues. Since potassium is especially important in conduction of nerve impulses and in their synaptic transmission, and since steroids of the type of desoxycorticosterone regulate potassium metabolism, it was thought that an investigation of the possible influence of pregnenolone and of desoxycorticosterone on potassium metabolism, especially of brain but also of muscle, would be of interest.

METHODS

Brain and muscle potassium values have been studied under a variety of experimental conditions in 300 male rats weighing between 100 and 140 grams. Groups of matched rats fed on Purina stock diet were compared as follows: normal rats vs. adrenalectomized rats, adrenalectomized rats vs. adrenalectomized rats injectioned with pregnenolone, normal rats vs. those given desoxycorticosterone, normal rats vs. those given pregnenolone, normal rats vs. those stressed by forced swimming for an hour, stressed swimming rats vs. those similarly forced to swim after previous pregnenolone injections. In addition, comparisons were made between brain and muscle potassium in rats stressed by other means.

Most of the analyses were done with a Perkin-Elmer flame photometer (1, 14, 23), although in some of the experiments radioactive potassium, K42, was used as a tracer.

In all but a few indicated experiments in which animals were killed by etherization, the rats were rapidly killed by decapitation; control rats alternating in all cases with experimental animals in the sequence of killing. Tissues, brain and muscle, were removed immediately from *alternating* control and experimental animals. The tissues were placed on filter paper on crucible covers and weighed at once in the wet state. Thus, in a typical experiment involving 20 animals, all were decapitated in a span of three or four minutes and all of the 40 muscle and brain samples were excised and weighed within an hour. The alternation of control and experimental sampling is, of course, important to prevent losses of weight by evaporation or other changes which might modify results in favor of either the control or of the experimental group.

The weighed tissue, whole brain or gastrocnemius muscle, was ashed at 550°C. for one and one half hours in a muffle furnace. In tests with K42 the ashing was done on a crucible cover which was then covered with thin tissue paper and inverted over the mica window of our Geiger-Muller counter.

In experiments with the flame photometer, the ash was taken up in 100 cc. of water, diluted one part to three, and aliquots of this sample were then analyzed for their potassium content and compared to frequently checked standard solutions. In all of the analytical procedures, we alternated control and experimental samples in making our determinations.

In experiments with K42, our results have been expressed as counts per minute

per gram of wet tissue and in the flame photometer experiments as mgm. of potassium per gram of wet tissue. In the section on "Discussion" our reasons for using a wet weight basis will be considered. In the final results, we have expressed the percentage change of brain and muscle potassium concentrations produced by the various experimental procedures and calculated the statistical significance of the differences.

RESULTS

Effects of adrenalectomy and of pregnenolone on brain and muscle potassium. Data were obtained on 38 male rats. Thirteen of them were used as normal controls, and nine additional unoperated animals were given 0.5 mgm. per day of pregnenolone acetate in oil subcutaneously for three days prior to sacrifice. Sixteen rats were adrenalectomized four days before sacrifice, and six of these were given daily injections of pregnenolone as in the case of the nine unoperated animals. The adrenalectomized rats were not compensated with NaCl.

All of the 38 rats were injected intraperitoneally with 3 cc. of a solution containing radioactive potassium (K^{42})³ 36 hours before sacrifice, a time interval adequate for the tracer to become distributed throughout the tissues according to data of Noonan, Fenn and Haege (22). The K^{42} solution contained 114 milliequivalents of K per liter as KCl at a pH of 7. The half life of K^{42} is 12.4 hours and when it was received from the Clinton pile the activity was such that a 3.0 cc. injection gave a count 36 hours later of approximately 6000 counts per minute per gram of wet tissue.

The rats were killed by etherization and the whole brain and one gastrocnemius muscle were removed from each animal. The tissue specimens were ashed on individual crucible lids and the counts were obtained on each of the individual specimens. Table 1 shows the results.

From the data it may be seen that adrenalectomy increases the concentration of muscle potassium by 37.5 per cent over that of normal animals. Harrison and Darrow (15) and Buehl and Turner (2) have also found increased muscle potassium in rats following adrenalectomy. According to Harrison and Darrow, rats, in contrast to dogs, cats and man, show little or no change in intracellular-extracellular water distribution in uncompensated adrenal insufficiency. We know of no work on the potassium content of brain in adrenalectomized animals. Our data show an increase of 24.0 per cent in the concentration of brain potassium four days after uncompensated adrenalectomy in contrast to an increase of 37.5 per cent in that of muscle.

In our experiments, it may be seen from table 1 that pregnenolone does not affect the potassium concentration of brain or muscle in normal rats but does prevent the marked rise in brain and muscle potassium concentration in adrenalectomized rats. Thus brain potassium concentration increases only 14.0 per cent (instead of 24.0 per cent) and muscle potassium 25.5 per cent (instead of 37.5 per cent) in adrenalectomized rats protected by pregnenolone. All of these differences are statistically significant.

³ The K^{42} was obtained from the Clinton pile through the Atomic Energy Commission.

Effects of desoxycorticosterone acetate (DCA) (Schering) on brain and muscle potassium and sodium. While pregnenolone does not affect the potassium concentration of brain and muscle of normal unstressed rats, desoxycorticosterone acetate (DCA) injected in the same amount and under the same conditions results in a small but significant lowering of muscle potassium concentration, although it has no significant effect on brain potassium. DCA is the adrenal steroid primarily effective in the control of sodium and potassium metabolism (4, 10; see Goodman and Gilman (11), Thayer (30) for reviews).

TABLE 1. COUNTS PER MINUTE PER GRAM WET WEIGHT TISSUE

	1	2	3	4	Comparing columns 2 to 1	Comparing columns 4 to 3	Comparing columns 3 to 1
	Normal control	Normal control pregnenolone in- jected	Adrenalectomized rats	Adrenalectomized pregnenolone in- jected			
Brain	4897 ±124	4802 ±200	6075 ±308	5460 ±360	-2% P not signif.	-10% P<0.01	+24% P<0.01
Muscle	5943 ±143	6123 ±108	8172 ±244	7198 ±381	+3% P not signif.	-12% P<0.01	+37.5% P<0.01

TABLE 2. MGM. POTASSIUM AND SODIUM PER GRAM WET WEIGHT TISSUE

	9 NORMAL RATS		9 DCA-INJECTED RATS		% CHANGE IN- JECTED VS. CON- TROLS	% CHANGE IN- JECTED VS. CON- TROLS
	K	Na	K	Na	K	Na
Brain	3.138	1.156	3.108	1.138	1.0% loss P not signif.	1.5% loss P not signif.
Muscle	3.164 ±0.06	1.002 ±0.01	2.967 +0.05	1.045 ±0.025	6.2% loss P = 0.02	4.0% gain P not signif.

Nine rats were injected with DCA in oil (0.5 mgm. per day for five days) prior to sacrifice, and nine paired rats were kept as controls. The animals were killed by decapitation and the brain and one gastrocnemius muscle from each rat were analyzed for potassium and sodium with the flame photometer. Table 2 shows the results.

The only significant effect is a decline in potassium concentration in muscle of 6.2 per cent. The 4.0 per cent gain in muscle sodium is not statistically significant. Miller and Darrow (4, 21) have also reported a loss of potassium from muscles of normal rats given DCA.

We have not examined the effect of DCA on brain and muscle potassium of adrenalectomized rats. Harrison and Darrow (15) have shown that adrenal cortical extract reduces the high muscle potassium of adrenalectomized rats to normal, presumably via its DCA-like components.

Stress of prolonged swimming in relation to brain and muscle potassium and the effect of pregnenolone on tissue potassium. From the data of table 1, it is clear that pregnenolone in the small amounts we used lessens the excessive accumulation of potassium in both brain and muscle resulting from adrenalectomy. In this way it acts like adrenal cortical extract which regulates electrolyte balance by way of its desoxycorticosterone-like content.

Because of our studies of psychomotor fatigue in man in relation to pregnenolone, we were particularly interested in effects of fatiguing stresses on brain and muscle potassium and possible influences of pregnenolone on tissue potassium in relation to stress. Was it possible that fatiguing stresses might modify tissue potassium and that pregnenolone would prevent such changes?

Miller and Darrow (21) stressed rats by forced swimming for periods ranging up to an hour. They found no loss of potassium from muscle as a result of the exercise, although it has been well established that isolated muscle stimulated to contract through its nerve loses potassium. (See Fenn (9) and Darrow (5) for reviews.)

We have stressed rats by forced swimming at 28°C. for an hour. In each experiment all rats, both of the experimental and control groups, were killed immediately after the swim by decapitation, and brain and muscle potassium were determined by the flame photometer. The variability of tissue potassium was found to be so great that it was necessary to use considerably larger numbers of animals than had been used by Miller and Darrow before statistically significant differences were obtained. As may be seen from table 3, there is a small but significant decrease in the concentration of potassium in both brain and muscle of the swim-stressed rats compared to the controls, i.e., 4.4 per cent decrease in brain and 6.1 per cent decrease in muscle.

A comparison of data from tables 2 and 3 show different values of potassium in the two groups of normal control rats. In each experiment we have used control rats to match against our experimental groups, since over the months occupied by the work we have found from time to time occasional differences in mean potassium analyses of tissues in different groups of rats. When the data of table 2 were obtained, our rat colony was suffering from a mild form of infectious diarrhea with some loss of appetite, and we think it likely that potassium loss in the stool may account for the low values of the experimental and control groups of table 2, in contrast to the higher values later obtained as shown in tables 3 and 4. Our interest throughout has been in the comparative values of potassium in relation to matched groups of rats and we have not attempted to do potassium balances. The data of tables 3 and 4 show better representative values of typical potassium levels than do those of table 2, but the comparison of relative potassium values of control and experimental animals we believe to be equally valid for the data of each table.

The effects of pregnenolone on potassium changes resulting from the stress of swimming is also shown in table 3. In these experiments a group of rats were injected with 0.5 mgm. of pregnenolone per day for four days prior to the swim. These rats, together with an uninjected matched control group, were all rapidly

TABLE 3. MGMS. POTASSIUM/GRAM WET WEIGHT TISSUE

	1	2	3	Comparing column 2 to 1	Comparing column 3 to 2	
	Normal control	Swim stress	Swim stress pregnenolone-in- jected			
Brain	3.530 ± 0.026	3.376 ± 0.036		-4.36% $P < 0.01$		54 swim stress vs. 65 normals. Combina- tion of 6 experi- ments.
		3.236 ± 0.05	3.336 ± 0.05		+3% $P = 0.1-0.2$	36 swim stress vs. 23 swim pregnenolone-injected. Combination of 2 experiments.
Muscle	3.356 ± 0.047	3.15 ± 0.05		-6.14% $P < 0.01$		32 normals vs. 29 swim stress. Com- bination of 5 ex- periments.
		3.198 ± 0.067	3.349 ± 0.072		4.7% $P = 0.1-0.2$	26 swim stress vs. 21 swim stress pregnenolone-injected. Combination of two experiments.

TABLE 4. MGMS. POTASSIUM/GRAM WET WEIGHT TISSUE

	1	2	Comparing columns 2 to 1	
	DCA-injected	DCA-injected swim stress		
Brain	3.471 ± 0.04	3.579 ± 0.03	+3.1% $P = 0.1-0.05$	1 experiment. 10 DCA-injected ani- mals (0.5 mgm/day for 5 days) vs.
Muscle	3.672 ± 0.04	3.396 ± 0.06	-7.5% $P < 0.01$	10 DCA-injected ani- mals (0.5 mgm/day for 5 days) swim stressed.

MGMS. SODIUM/GRAM WET WEIGHT TISSUE

Brain	1.176	1.182	+0.5% P not signif.	
Muscle	0.776 ± 0.016	0.845 ± 0.02	+9% $P = 0.02-0.05$	

decapitated at the end of the swim of the experimental group, and the brain and muscle potassium values were individually determined with the flame photometer.

Pregnenolone-injected rats that were forced to swim have a 3.0 per cent higher potassium concentration in their brains and 4.7% higher potassium concentration in their muscles than have rats forced to swim but not previously injected with pregnenolone. These differences are in the right direction if pregnenolone prevents loss of potassium due to the stress. However, the results are not statistically significant. The P value of 0.1 to 0.2 indicates that there is a ten to 20 per cent likelihood that the differences are due to chance.

Sodium values were also obtained in the experiments involving swimming, but they were highly variable. No significant differences were observed in the sodium content of the brains of rats forced to swim as compared to unstressed controls, but a significant 23 per cent increase in the concentrations of muscle sodium ($P \pm 0.01$) was found in rats forced to swim corresponding to a decline of 6.1% of potassium concentration in this group.

If the decline in concentration of potassium in brain and muscle is important in the development of fatigue, and if pregnenolone tends to prevent this decline, it should follow that pregnenolone-injected rats would be able to swim longer than controls.

The time that a rat can swim before exhaustion is dependent upon the temperature of the water. While most rats are not exhausted after an hour's swim at 35°C., few rats fail to drown in half an hour in water at 15°C.

Eighteen rats were forced to swim in a bath at 22°C. Nine of them had previously been injected for five days with 0.5 mgm. per day of pregnenolone in oil. The nine control rats were given placebo injections of oil primarily so as to be comparable in buoyancy to the pregnenolone group. The period from the start of the swim to drowning was measured. The average length of swim of the pregnenolone group was 56 minutes, and that of the control group 41 minutes, but the variability was so great (from 14 minutes to 180 minutes) that this result is not statistically significant. We estimated that at least several hundred rats would require drowning adequately to answer this question and our colony could not at the time afford this attrition rate.

Effect of DCA on potassium metabolism under conditions of swim-stress. From the data of table 2, it may be seen that normal unstressed rats injected with DCA show a reduction of 6.2 per cent in the concentration of muscle potassium.

Table 4 indicates that DCA injected into rats at a rate of 0.5 mgm. per day for four days prior to forced swimming produces a drop in the concentration of muscle potassium following the swim below the levels found in uninjected swim-stressed rats. There appears to be no compensatory effect of the hormone in overcoming the decline in potassium concentration from muscle due to stress as is suggested from the results with pregnenolone. Accompanying the decline in potassium concentration is a significant (9 per cent) rise in the concentration of muscle sodium.

Animals previously injected with DCA and forced to swim, however, appear to show less of a decline of brain potassium concentration (by 3.1 per cent) than uninjected swim-stressed rats, and this difference is just barely significant. No difference in brain sodium is seen in the two groups. Pregnenolone and DCA thus both appear to act to prevent the lowering of potassium concentration in brains of stressed animals. Pregnenolone also prevents decrease in concentration in potassium from muscle, while DCA accentuates the decrease. But these effects are small and some of them may be due to chance. Figure 1 summarizes the data of the four tables.

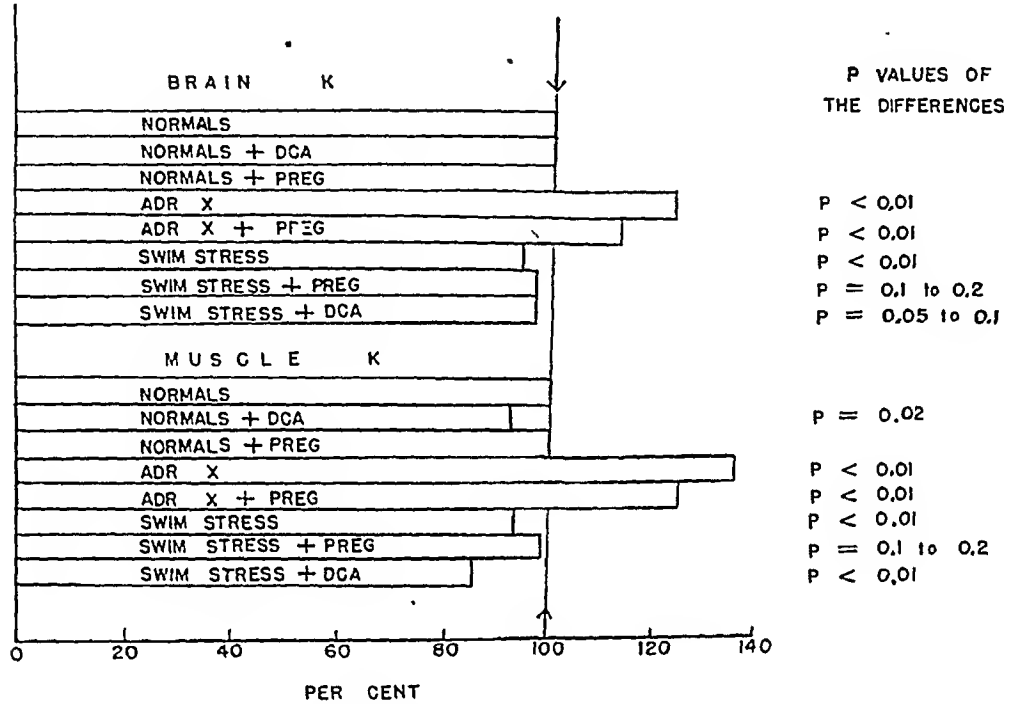


FIG. 1. SUMMARY OF THE DATA OF THE FOUR TABLES. Per cent change (wet weight basis) of potassium concentration in brain and muscle under various experimental conditions.

Other stresses. Work from our laboratory (7) has demonstrated that exposures of rats to cold and also to the stress of tying them to a grid markedly excites the adrenal cortex as indicated by pronounced lymphocytopenia. This lymphocytopenia is absent in comparably stressed adrenalectomized rats. Dougherty and White (6) have demonstrated that lymphocytopenia occurs when adrenal cortical extract or adrenocorticotrophin is injected into normal rats, and that adrenocorticotrophin fails to produce lymphocytopenia in adrenalectomized rats. More specifically, they have shown that the drop in lymphocytes results from the action on lymphoid tissue of 11-oxygenated, 'sugar' steroid hormones from the adrenal cortex.

In view of the excitation of the adrenal cortex by cold stress and grid stress, we felt it would be interesting to examine brain and muscle potassium and sodium in relation to these stresses. Twenty rats were exposed in a cold room to a

temperature of 2°C. for four hours, immediately decapitated and their brains and muscles analyzed for potassium and sodium with the flame photometer. Twenty paired rats, kept at room temperature, were used as controls. No significant differences were found in brain or muscle potassium and sodium between the experimental and control groups.

Ten rats were tied to a wire grid for two hours at room temperature and their brain and muscle sodium and potassium values were compared to those of the unstressed control rats. We found no differences in brain or muscle sodium or potassium between the two groups.

Our failure to find changes in the concentration of tissue potassium with cold and grid stresses in contrast to our findings with the stress of swimming suggests that a possibly different type of excretion of adrenal cortical hormones may be involved in the two cases. This is borne out by our observations (to be published elsewhere) that the stress of swimming produces marked lymphocytosis in contrast to the equally marked lymphocytopenia resulting from cold or tying to a grid. It is possible that different pituitary corticotropins may be involved in the release of different steroid hormones from the adrenal cortex in the case of the different stresses.

DISCUSSION

Zwemer and Truszkowski (31) have emphasized the similarity of symptoms, including fatigue symptoms, between adrenal insufficiency resulting in potassium retention and the symptoms of potassium poisoning. From our experiments there is a reduction of brain and muscle potassium concentration with prolonged activity. Pregnenolone in small doses appears to have a compensatory action on both increased potassium concentration in adrenal insufficiency and decreased potassium concentration due to stress.

This homeostatic action of pregnenolone on potassium, particularly in relation to the brain, is suggestive in connection with the action of pregnenolone in enhancing the efficiency of prolonged psychomotor behavior in man.

We have expressed our potassium analyses on a wet weight basis, despite well established demonstrations by others (5) of changes in water content of muscle with activity. The analysis of some 600 tissue samples has been facilitated by elimination of the operation of drying the tissue, but in addition we have felt that the concentration of potassium per gram of tissue, despite its water content, is of as much, if not more, physiological interest than is the amount of potassium per unit weight of solids. Potassium greatly modifies excitability of tissues including nerve, and depending upon concentration gradients, it can either enhance or inhibit excitability (see Fenn (9) for review). Entrance or loss of water modifies potassium concentration as does the loss or entrance of potassium itself.

Fenn (9) has reviewed evidence indicating that nerve together with many other excitable tissues loses potassium during activity, and he has reviewed the demonstrated relationship between the metabolism of potassium and that of acetylcholine in nerve conduction and synaptic transmission. Potassium is highly

mobile in protoplasm and nerve cells, like most other cells, contain a high inside concentration of potassium amounting to 10 or 40 times that in the surrounding fluids. Hoagland (17) has demonstrated that the excitability to mechanical stimuli of single nerve endings in the frog skin is determined by the ratio of concentration of potassium inside the fiber to that outside. This ratio of potassium inside to potassium outside (K_i/K_o) appears to result in major part from the intracellular formation of H^+ produced by the metabolic formation of acid substances, especially CO_2 and the exchanges of H^+ for K^+ (See Grundfest (13) for review).

Curtis and Cole (3), following leads from Osterhout's (24) work with 'impaled' *Valonia* plant cells, succeeded in pushing a microelectrode into the axoplasm along the axis of a giant nerve fiber of the squid, so that the tip was just opposite the outside electrode. The membrane potential then measured was 50 mV. Upon raising the outside concentration 18-fold (making K_i/K_o zero), the potential was reduced to zero, and upon raising it 40-fold, the potential was reversed 15 mV. Similar results have been obtained by Graham, Carlson and Gerard (12) for single frog muscle fibers.

Since the electrical properties of the cell depend upon this ratio rather than upon the absolute potassium content, and since K_i/K_o can be modified by movement of either water or potassium, we have expressed our results on a wet weight percentage basis rather than on a dry weight basis. There seems to us to be little choice between expressing potassium values on a fat-free dry weight basis or on a wet weight basis. K_i/K_o is lowered if potassium is lost from the cell. It is also lowered if water enters and lessens the internal concentration. Since little is known about the distribution of intracellular, extracellular and bound water in muscle under different experimental conditions, and since nothing is known about this distribution in brain, we believe that the wet weight concentrations of potassium are as meaningful as a first approximation as are concentrations expressed on a dry weight basis. It follows, of course, that neither is necessarily meaningful.

Recently Hodgkin (18) found that small changes in external potassium concentration caused large and rapidly reversible changes in the conductance of crab nerve membranes. Hodgkin and Huxley (19) have calculated that 1.7×10^{-12} moles of potassium leak through one square cm. of crab nerve membrane per impulse and that 3×10^{-10} mol. cm.⁻² sec.⁻¹ are reabsorbed when the external concentration of potassium is three times its normal value.

Potassium in nerve, because of its high mobility and consequent internal accumulation, is important in determining nerve excitability and in the conduction and transmission of nerve impulses. The fact that other cations, notably rubidium, not present in body fluids in appreciable amounts, affect the electrical behavior of nerve in a way similar to that of potassium (20) does not militate, in our opinion, against this view. Homeostasis in the concentration of brain potassium would thus be expected to be associated with optimal brain functioning, and it is not unreasonable to expect modification of the conduction of impulses in the central nervous system if brain potassium concentration is either elevated or depressed.

Heppel (16) studied changes in water, chloride and potassium content of rat gastrocnemius muscles when made to contract by electrical stimulation of the sciatic nerve. He confirmed previous work of Fenn and co-workers (9) on muscles of rats and other animals, indicating that stimulation of muscles results in a loss of potassium in exchange for sodium and a gain in NaCl and water. Heppel found that the loss of potassium on stimulation is greater the greater the duration of contraction up to 30 minutes, with a decline in rate of loss after this time. The changes in water and chloride were maximal after five minutes of stimulation and much reduced at the end of an hour. While the water variations parallel those of chloride, potassium loss was not correlated with the water changes. The feeding of diets low in potassium had little or no effect on the potassium changes during stimulation.

Fenn (8) earlier had studied the effect of voluntary contraction of muscles in nine rats forced to swim to exhaustion. He cut one sciatic nerve in each animal and compared the potassium and water content of the active and inactive leg muscles. He summarized his results as follows:

"Rats with one sciatic nerve cut were exercised by swimming. The muscles sampled immediately afterwards and analyzed showed that voluntary contractions produced an increase of water content and a decrease of potassium (on a dry weight basis). In general the muscles of rats which swam the longest and were least quickly fatigued lost the most potassium but gained the least water. Muscles sampled a few days after denervation showed a slight gain in potassium, presumably because of the lack of activity."

Miller and Darrow (21) were unable to find any loss of potassium from rat muscles (dry weight basis) after the voluntary exercise of forced swimming, although an elevated serum potassium was found after the exercise. In one group of experiments they lowered the potassium content of muscle potassium in rats by feeding them on a potassium-free diet, and in another group they raised the potassium content by injecting the animals with potassium chloride. Within wide limits they found that potassium in muscle cells does not limit the capacity of rats to swim continuously for 60 minutes. They criticized Fenn's findings on the grounds that his reported post-swim loss of potassium from active muscles was not statistically significant.

Miller and Darrow (21) found that the injection of two to 4 mgm. per day of DCA for nine to ten days in eight rats gave an average muscle potassium content of 40.5 mM potassium per 100 gram fat-free solid, which contrasted with a mean value of 48.8 in a group of 13 uninjected rats. The difference of 8.3 mM of potassium corresponds to a loss of 17.0 per cent in muscle potassium in the DCA-injected group, and this may be compared to the 6.2 per cent loss of potassium we found on a wet weight basis after injecting 0.5 mgm. per day of DCA for five days (table 2).

Miller and Darrow (21), however, report that the stress of swimming raises the level of potassium in muscles of DCA-injected rats, and this appears to be contrary to our results since we found an apparent loss of muscle potassium in DCA-injected rats that were forced to swim. In the case of brain, however, DCA tends to restore to a normal level the decrease in concentration of brain

potassium resulting from forced swimming (table 4), and pregnenolone appears to do this also for muscle as well as for brain (table 3).

Our results show that both brain and muscle display a significantly decreased concentration of potassium after forced swimming, and thus we are in agreement with Fenn's finding regarding changes in muscle potassium with voluntary exercise. Our findings appear to be in disagreement with the results of Miller and Darrow in this regard, although it was necessary to use many more animals than other investigators had used to demonstrate the change in potassium concentration with statistical validity. Since we have expressed results on a wet weight basis, it is possible the apparent potassium loss we note with exercise in muscle is due to a gain in water. For reasons discussed above we believe that potassium concentration changes, whether they be due to gain or loss of either water or potassium, is of primary physiological interest.

SUMMARY

1. Studies of the concentration of brain and muscle potassium of 300 rats were made under a variety of experimental conditions.

2. Small (0.5 mgm. per day) doses of desoxycorticosterone or of $\Delta 5$ -pregnenolone did not affect brain potassium in normal rats; desoxycorticosterone, but not $\Delta 5$ -pregnenolone, reduced the concentration of muscle potassium by 6.2 per cent.

3. Uncompensated adrenalectomy increased the concentration of brain potassium by 24.0 per cent and of muscle potassium by 37.5 per cent.

4. $\Delta 5$ -Pregnenolone (0.5 mgm. per day) lessened the increase in concentration of brain potassium in adrenalectomized rats by 10.0 per cent and of muscle potassium concentration by 12.0 per cent.

5. The stress of prolonged swimming reduced the concentration of brain potassium by 4.4 per cent and that of muscle by 6.1 per cent. These, together with the foregoing results, were all statistically significant ($P < 0.01$).

6. There was a suggestion that doses of 0.5 mgm. per day of both $\Delta 5$ -pregnenolone and desoxycorticosterone tended to prevent the loss of brain potassium due to prolonged swimming, although the results were not statistically significant. (P approximately 0.1.)

7. $\Delta 5$ -pregnenolone also tended to prevent the decrease of potassium concentration in muscles of animals forced to swim, but the P value was not significant (0.1 to 0.2).

8. Desoxycorticosterone significantly augmented the decrease of concentration of muscle potassium in swim-stressed rats.

9. The results are discussed in relation to effects of potassium on tissue excitability and to the antifatigue action of pregnenolone.

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CONDUCTION RATES AND DORSAL ROOT INFLOW OF SENSORY FIBERS FROM THE KNEE JOINT OF THE CAT^{1, 2}

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Received for publication September 2, 1947

The analysis of the neural control of skeletal muscle as it acts on diarthrodial joints has been carried to a very high degree by Sherrington and many subsequent workers, but surprisingly little attention has been paid to the innervation of joints themselves, either from an anatomical or physiological standpoint. This is particularly astonishing in view of the incidence of crippling joint disorders in which nerves must be implicated since severe pain is a common symptom. Yet it is not known just where such pain arises. Raszeja and Billewicz-Stankiewicz (10) described areas on the medial and lateral sides of the rabbit knee joint, the stimulation of which, with a faradic current of two seconds' duration, was followed by responses which indicated that the stimulus was painful. Leriche (9) on the other hand stated that an anesthetic injected into the cavity of a painful joint had no effect on the pain, but when it was injected into the external part of the capsule and around the joint, the pain was relieved. Our knowledge of the transmission of pain from joints is no more precise than indicated in these two reports.

Position sense is a useful test in neurological examinations and undoubtedly an important quality in postural and locomotor mechanisms. It seems highly probable that stimulation of joint receptors is one of the necessary components. This was borne out by Stopford (12) in his studies of peripheral nerve lesions. He pointed out that the so-called cutaneous nerves of the fingers give branches to the interphalangeal joints. When these nerves are destroyed the patient can still detect joint movements passively induced by the examiner, but his localization of direction, degree and site of movement is greatly impaired. Stopford felt that recognition of movement as such could result from changes in tension of tendons, the nerves to which were still intact. Presumably, then, joint endings are stimulated during movement. Barnes (2) recorded potentials from sensory nerves of crustacean limbs while the joints of such limbs were being bent. In a search of the literature, however, no reports of similar studies in vertebrates were found.

There is considerable clinical evidence that the central pathway for position sense is by way of the dorsal funiculus, medial lemniscus and internal capsule, but there have been no experimental studies of any of the possible central connections of these receptors, aside from some studies by Adrian on cerebellar action potentials (1). He recorded potentials during various types of peripheral stimuli, among which were joint movements.

¹ The initial phases of this work were presented at the Annual Meeting of the American Association of Anatomists at Montreal, April 1947.

² Aided by a grant from the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service.

There are only scattered reports relative to reflexes in which these nerves and their terminals may partake. Harrison *et al.* (7) and Comroe and Schmidt (4) in their studies of respiratory mechanisms concluded that impulses from joint receptors initiated reflex increases in respiration. There have been no studies of possible reflex effects on skeletal muscle.

Our lack of knowledge is further emphasized by the fact that, so far as human joints are concerned, no one has determined exactly what kinds of nerve endings are present. The evidence that Pacinian corpuscles are found in joint capsules is quite unsatisfactory (5).

There are other points which could be made but these are enough to indicate how little attention has been paid to the functions of normal joints.

Distribution of nerves to the knee joint. An experimental approach to these problems depends upon a rather exact knowledge of the anatomy of joint nerves. Because of this it was felt that studies of the cat would be most satisfactory since it has been shown that the nerves to the knee joint of this animal are distributed in a definite pattern and terminate in various types of receptors in the joint capsule (5). The joint is supplied mainly by the femoral and tibial nerves, and to a lesser extent by the obturator. Two branches to the joint are larger and more constant than any others. One arises from the tibial nerve and, because of its position relative to the knee joint, is designated the posterior nerve. The average nerve contains approximately 170 myelinated fibers which range from two to 16 microns in diameter. The majority are either in a two to five or seven to 10 micron group, the latter containing the greater number of fibers. In addition, there are about 115 nonmyelinated fibers.

The other major branch to the joint is designated the medial nerve. It usually arises from the saphenous branch of the femoral nerve, but occasionally from the obturator, or from both. The average nerve contains approximately 145 myelinated fibers which range from two to 16 microns in diameter. Like the posterior nerve, most of the fibers are either in a two to five or seven to 10 micron group, but in this nerve the two to five micron group contains the greater number of fibers. In addition, there are about 120 nonmyelinated fibers.

METHODS

There were several questions, the answering of which constituted the initial studies of these nerves and their endings. What are the conduction rates of these articular fibers and do they correspond with measured diameters? Are the fibers efferent or afferent, and if the latter, in which dorsal roots do they enter the spinal cord? What are the effective stimuli for the receptors which many of these fibers form?

Adult cats were anesthetized with sodium pentobarbital given intraperitoneally. In approaching the posterior nerve the gastrocnemius muscle was split and the tibial nerve exposed. This was traced towards the popliteal fossa, at the lower part of which there are several branches distributed mainly to the neighboring muscles. The first of these branches is usually the posterior nerve. It descends in the sheath of the tibial nerve for a short distance and then leaves it, running a recurrent course towards the back of the joint capsule, crossing the

popliteal vessels as it does so. Its subsequent distribution has already been described in detail (5). The nerve was carefully cleaned of surrounding connective tissue, using glass hooks and probes for most of the dissection. In all cases the tibial nerve was sectioned distal to the origin of this branch so as to facilitate manipulation.

The medial nerve was approached by making an incision in the sartorius muscle in a line parallel to the more deeply placed vastus medialis, along which the nerve descends. The dissection was more difficult since the nerve is smaller than the posterior nerve, often courses as two fascicles and is always closely associated with or actually in the adventitia of the accompanying vessels. Because of these factors, in several dissections the nerves did not survive.

Types of experiments. The following experiments were carried out.

1. Dorsal roots were cut at their entrance into the spinal cord. Potentials were recorded from articular nerves as various roots were stimulated (fig. 1A). At first, several attempts were made to record from dorsal roots as articular nerves were stimulated, but it was found that the large number of fibers derived from areas other than the knee joint shunted out the desired potentials to a large extent. The antidromic method proved much more satisfactory on this account. Furthermore, the absence of a marked connective tissue sheath helped reduce shock artifact.

2. The sciatic and femoral nerves were ligated, cut centrally and all branches severed except the articular nerves. They were then stimulated and muscle contractions in the neighborhood of the joint closely watched for. Next the articular nerves were severed from the capsule, placed on electrodes and potentials recorded during stimulation of the parent trunks (fig. 1B). In this way articular fibers were activated and the recorded potentials could be compared with those obtained by stimulation of some of the same fibers in a particular dorsal root.

3. The articular nerves were severed from the parent trunks and placed on electrodes. Since they were still attached to the joint capsule, the method allowed the recording of any potentials originating from the stimulation of joint receptors.

The animals were kept warm but no special precautions were taken in regard to temperature control. The conduction distances were long and most of either the sciatic or femoral nerves were untouched and therefore at body temperature in those experiments in which conduction rates were measured. Exposed nerves were constantly kept moist with warm Ringer's solution. Under such conditions recordings were carried out over many hours without appreciable changes being observed.

The stimulating electrodes were silver wires, and a silver wire was also used to ground the nerves between stimulating and recording electrodes. The latter were glass tubes filled with Ringer's solution from which woolen wicks protruded and into which dipped chlorided silver wires. The nerves were slung on glass hooks and contact made with the moist wicks of the electrodes. In the earlier experiments, the stimulus was a condenser discharge, the duration of which was approximately one tenth of a millisecond. Later a thyatron control was substituted for the mechanical switching and this allowed considerable variation in both

frequency and duration of stimulus. In both cases the stimulus was applied through a shielded transformer which helped reduce shock artifact. The artifact was very marked in several cases, the cause being either a poor contact at the ground electrode or an accidental grounding of some other part of the animal,

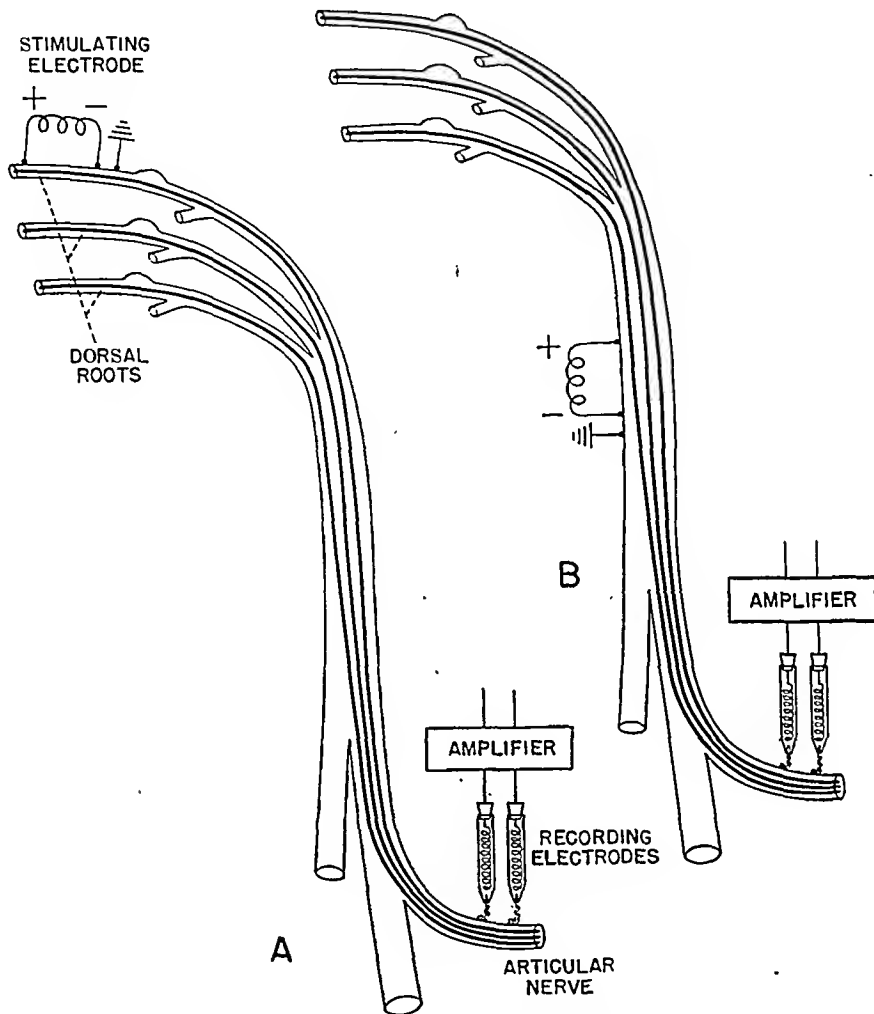


FIG. 1. *A*, DIAGRAM OF THE METHOD USED IN DETERMINING CONDUCTION RATES and dorsal root inflow. The main trunk represented is the sciatic, but the method was the same for the femoral nerve. *B*, Diagram showing how stimulation of the main trunk activates all the myelinated fibers from the articular nerve.

thus giving a double ground. Sixty-cycle interference often appeared in the latter situation. Both pickup electrodes were above ground, being connected to the input grids of a push-pull amplifier. The input was differentially arranged, with a high resistance common to both cathodes. The amplifier was resistance-capacity coupled with a time constant of about one and one-half seconds. Its high frequency response fell off sharply above 1000 cycles. The output was fed into a cathode ray oscillograph, the sweep of which was tripped by the stimulus.

RESULTS

Action potentials were successfully recorded in 19 of 24 cats. In 12 of these 19 the posterior nerve was studied and, in seven of these, photographic records were made. The medial nerve was studied in the remaining seven of the 19 cats and, in five of these, photographic records were made.

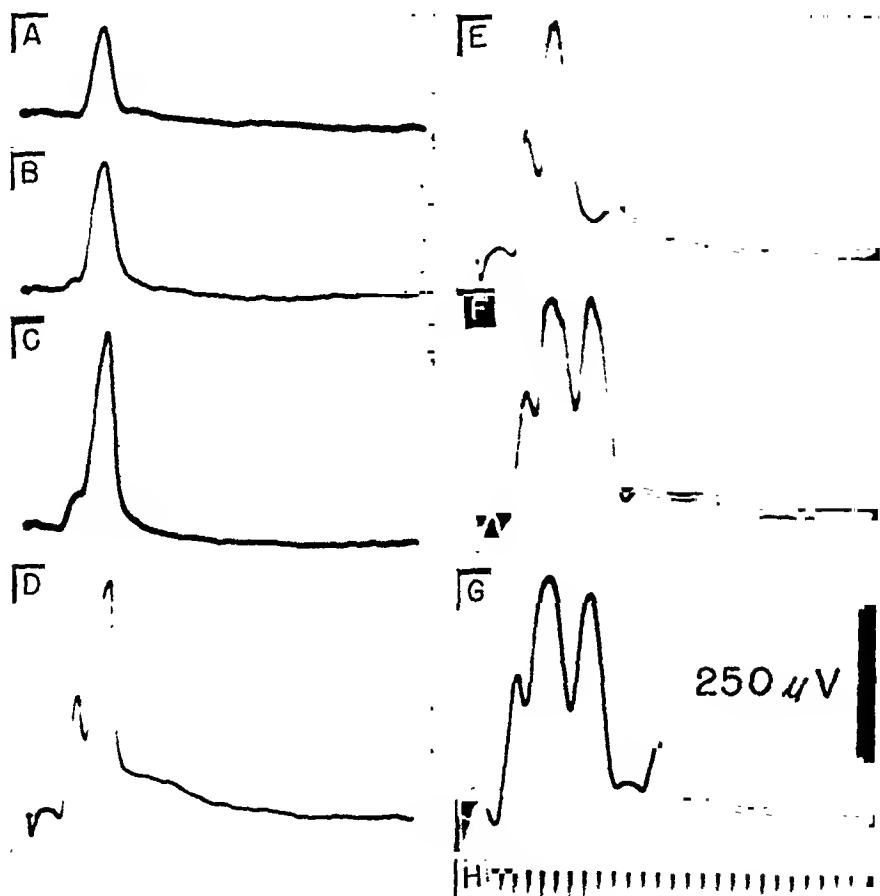


FIG. 2. POTENTIALS RECORDED FROM THE POSTERIOR NERVE DURING STIMULATION of the 7th lumbar dorsal root by the method of figure 1A. A-G. Responses at increasing strengths of stimulation, with development of characteristic major deflections. Note that the fast initial component does not have the lowest threshold. It is first seen in B at stimulus strength higher than that which elicited the response in A. H. Time in milliseconds.

Posterior nerve. Potentials were consistently recorded from the posterior nerve on stimulation of the 6th and 7th lumbar dorsal roots, and, in two cases, of the 1st sacral dorsal root as well. The conduction distances ranged from 16.5 to 18.5 centimeters. Consequently, the temporal dispersion was so great that the form of the potentials was often complex. At least two major deflections were usually recorded (fig. 2). In the first there was frequently a small initial component conducting at about 70 to 90 meters a second, but most of the elevation represented fibers conducting at approximately 40 to 60 meters a second. The second major deflection represented fibers conducting at rates of approximately 20 to 40 meters

a second. Following this were minor deflections of variable form and amplitude (figs. 2G and 3A), the slowest of which conducted at about ten meters a second.

The recorded potential was much larger when the sciatic was stimulated, since many more articular fibers were activated (fig. 1B). The conduction distance was shorter, so that the major deflections were combined into one, and the more slowly conducting fibers were represented by a somewhat complex and smaller terminal deflection (fig. 3B). When the articular nerve was left attached to the

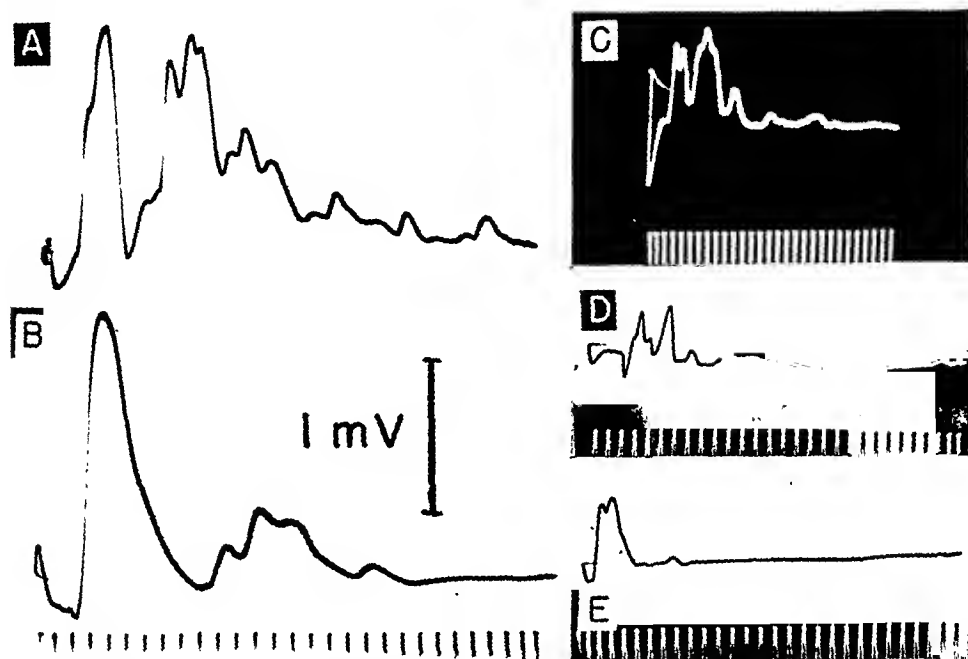


FIG. 3. A. POTENTIALS RECORDED FROM THE POSTERIOR NERVE DURING STIMULATION of 6th lumbar dorsal root. Contrast the number of slower waves with the single one in figure 2G. Some, however, may well be repetitive discharges from faster fibers as a result of relatively high stimulus strength. B. Potentials recorded from the posterior nerve during stimulation of the sciatic nerve by the method of figure 1B. As a result of the shorter conduction distance there is but a single major deflection. C-D. Potentials recorded from the medial nerve during stimulation of the 6th and 5th lumbar dorsal roots, respectively, showing the major deflections. Amplification approximately the same as for figure 2. E. Potentials recorded from the medial nerve during stimulation of the saphenous nerve showing but a single major deflection. Amplification approximately the same as for figure 3B. Time in milliseconds.

joint capsule during stimulation of the sciatic, muscular contractions were never seen, providing all other branches of the sciatic had been cut.

Medial nerve. In the case of the medial nerve, potentials were consistently recorded during stimulation of the 5th lumbar dorsal root, and, in three cases, of the 6th lumbar root as well. (In one case the femoral nerve was derived entirely from the 5th lumbar spinal nerve.) As in the case of the posterior nerve, the conduction distances were long, ranging from 14 to 19 centimeters. Again the temporal dispersion was great and the form of the potentials often complex. As a rule there were at least two, and sometimes three, major deflections (fig. 3C and

D). In all cases most of the first deflection represented fibers conducting at 30 to 60 meters a second, while in two cases there was a small initial component at 70 to 80 meters a second. The second major elevation was usually as large or larger than the first, and represented fibers conducting at rates of 20 to 30 meters a second. Succeeding this were either a single major deflection, or else variable smaller ones, the slowest of which conducted at nine or ten meters a second.

When the medial nerve was intact and the saphenous stimulated, no muscle contractions were ever seen, providing all other branches were cut (the branch of the femoral nerve to the pectineus may arise in conjunction with or directly from the saphenous). When the medial nerve was placed on electrodes and the saphenous stimulated, many more articular fibers were activated. Again, the potentials were larger, and, because the conduction distances were shorter, the major deflections were combined into one (fig. 3E). In one instance no potential was recorded during an experiment of this type, even though it had been successful during dorsal root stimulation. It was found that the medial nerve crossed over the saphenous and then pierced the adductor longus muscle and joined the obturator nerve. Subsequently it was found that the obturator sometimes gives a branch which joins the medial nerve, even when the latter is derived mainly from the saphenous.

DISCUSSION

The following conclusions may be drawn from these results.

1. Most, if not all, of these myelinated fibers are *A* fibers and are afferent in nature. If any are efferent, they would supply skeletal muscles in the neighborhood of the joint, and contractions of these muscles would then have been visible when the fibers were stimulated. This was never observed. If efferent fibers are present they must be nonmyelinated. It is quite likely that postganglionic vasomotor fibers may be included in this group.

2. It seems probable that conduction rates correspond to fiber diameters but there are two main reasons why a close fit cannot be made. The measurements of diameters were not made on the same nerves as those from which the potentials were recorded. Also, most of the determinations of conduction rates were made from records obtained during stimulation of dorsal roots. Since the articular fibers enter over several dorsal roots, there is no way of knowing the sizes of the fibers in a particular root. Fairly direct comparisons, therefore, can be made only between the largest fibers (about 16 microns in diameter) and the fastest rates (about 90 meters a second), and also between the smallest myelinated fibers (about two microns in diameter) and the slowest rates (about ten meters a second). According to Hursh (8) the ratio of diameter to conduction rate is a factor of six (using outside diameter, in contrast to the method of Gasser and Grundfest, 1939). This ratio is approximately realized in the instances cited here.

It is not unlikely that the ratio holds equally well for the other fibers and rates in which case fibers in the seven to ten micron group account for the first major deflections in the articular nerves. This group is more prominent in the posterior nerve and the corresponding potential is larger than any others. The suc-

ceeding major deflection in both nerves is probably obtained from fibers in the small, intermediate five to seven micron group and the two to five micron group. The latter is more prominent in the medial nerve and the corresponding potential is usually as large or larger than the first one.

In nearly all experiments, when dealing with threshold stimuli, there was a characteristic play of spikes, as described by Blair and Erlanger (3). The shock-spike distance varied from time to time even though the stimulus strength remained the same. Some of the variability may be attributed to utilization time, but in all cases the stimulus duration was much shorter than the shortest shock-spike distance observed under these conditions. Hence the variability is chiefly a result of changes in latency. In several experiments it was noticed that at threshold shocks the first spike to appear conducted more slowly than others which appeared with slightly stronger stimuli (fig. 2A and B). At the strengths used it is unlikely that this represents spread of excitation from the cathode, but rather that these were real differences in threshold, with a few fibers having a lower threshold than others with a slightly faster conduction rate.

3. The afferent fibers enter the spinal cord over several dorsal roots and the range of entry is surprisingly wide. The minimum is three dorsal roots, the 5th, 6th and 7th lumbar. In some cases the 1st sacral would be included as well, making four roots over which a total of approximately 315 myelinated fibers enter the spinal cord. Since the 6th lumbar dorsal root always contains fibers from the posterior nerve, and often from the medial as well, this root may well have the heaviest number of entering articular fibers.

In no instance were slow potentials of the *C* fiber type recorded, although they were searched for with very slow sweeps and maximum amplification. There are several possible explanations for this. One is that the potentials became so dispersed over the long conduction distances that they fell into the noise level of the amplifier (one to two microvolts). Others, however, have recorded *C* potentials after long conduction distances (11). It is also theoretically possible that the nonmyelinated fibers in these nerves are all autonomic, but it would be most surprising if this turned out to be the case. Probably the stimulus time used throughout these experiments was too short (one tenth to one millisecond), in which case either shocks of longer duration or greater strength or both should have been used. It is hoped that this point will be settled in future studies.

4. As yet, little can be said regarding the functions of these nerves and their endings. The nonmyelinated fibers form free endings in association with blood vessels and in the connective tissue of the capsule, particularly near Ruffini-type endings. The myelinated fibers form free and simple branched endings in connection with blood vessels, in the connective tissue of the capsule, and the larger ones form Ruffini-type endings in the posterior capsule at the zone of entrance of the posterior nerve (5). This is the portion of the capsule which is compressed during flexion of the knee joint. When the articular branch is severed from the tibial nerve, but left attached to the joint capsule, spontaneous potentials can be recorded (fig. 4). The causative stimuli have not been studied in detail, but it is possible that their origin is some stimulation of the Ruffini-type endings in the

posterior capsule. Whether this is a result of tension on the capsule by attached muscles remains to be determined. The potentials probably do not arise from the cut end of the nerve because difficulty of this sort was not encountered in other methods of recording. Furthermore, the potentials disappear when the nerve is cut or crushed between the electrodes and the joint. Most significant, however, is the fact that there is a definite increase in the frequency of these potentials when the posterior capsule is pressed lightly with a glass rod. Bursts of impulses were elicited by such a maneuver. Incidental observations indicate that these receptors are slowly adapting, but as yet the exact time of adaptation is unknown.



FIG. 4. A-B. SPONTANEOUS POTENTIALS RECORDED from the posterior nerve without pressure applied to the capsule. C-D. Similar potentials recorded from a different posterior nerve. E-F. Same nerve as C-D, showing definite increase in frequency of potentials when light pressure is applied to the capsule.

SUMMARY

It has been determined that myelinated fibers in articular branches of the tibial, saphenous and obturator nerves are A fibers, that their conduction rates seem to correspond with the ranges of fiber diameters usually found in these nerves and that they are afferent in nature. They enter the spinal cord through the 5th, 6th and 7th lumbar dorsal roots, and sometimes the 1st sacral as well. There is no evidence that any of the fibers are motor, but the probability that many of the nonmyelinated fibers are vasomotor is not eliminated. Finally, it is presumed that the large spontaneous potentials which can be recorded arise from stimulation of Ruffini-type endings in the posterior capsule, because pressure in this region increases the frequency of the potentials.

The author is indebted to Dr. J. D. Green for valuable assistance and helpful criticism during the course of the work. He further wishes to thank Mr. Bernard Agranoff for aid in the photography and Misses Evelyn Erickson and Geraldine Chesney for the preparation of the illustrations.

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URINARY EXCRETION OF ASCORBIC ACID BY GUINEA PIGS WITH HEALING SKIN WOUNDS

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Received for publication December 24, 1947

The necessity of ascorbic acid for the synthesis of collagen is now generally recognized (1, 2). Its rôle in the healing of wounds is presumably related chiefly to this function (3-6), though whether the vitamin serves as a building stone or as a catalyst in collagen formation has not yet been demonstrated. Convincing evidence has been presented of greater retention of ascorbic acid following injury as will be noted in the following:

Lauber and Rosenfeld (7) found that ascorbic acid tends to concentrate in and around wounded areas. Bartlett, Jones and Ryan (8) confirmed these results and showed in addition that the content of the vitamin in the wounded area varies with the ascorbic acid intake. They found as much as 6.53 mgm./100 grams in scar tissue of guinea pigs on a preoperative and postoperative diet high in vitamin C.

Andrae and Browne (9, 10) have recently reported results of a study of ascorbic acid metabolism after trauma in man and found that all of the injured subjects showed a low urinary output with large doses of ascorbic acid and that the retention was greater after burns than after fractures. Following the injury the level in the blood fell markedly and failed to rise after 15 to 20 days on the high intake suggesting destruction or utilization of the vitamin. A high ascorbic acid retention lasting only one to two days similar to that in normal individuals was observed several months after recovery from the injury. The evidence was considered inconclusive as to whether the ascorbic acid is destroyed or is incorporated in the newly-formed tissues. The fact that the retention was greatest immediately after the injury and became less so later when the tissue anabolism was increased suggested that the vitamin was not being used up in the formation of new tissues.

Levenson *et al.* (11) determined the plasma level and urinary excretion of ascorbic acid in six patients with severe surgical conditions to whom ascorbic acid was administered intravenously. They found abnormally small amounts in both blood and urine during the period of acute illness. Their findings were interpreted as affording no evidence as to whether or not the vitamin serves a useful purpose during its disappearance.

The need of further study of the rôle of ascorbic acid in wound healing is evident, particularly with the objective of determining whether any of the vitamin is utilized in anabolic processes.

EXPERIMENTAL METHODS

Adult male guinea pigs of an inbred strain¹ which had been kept saturated with ascorbic acid since shortly after weaning were used as test animals. Animals were selected which had shown a moderately steady excretion over a long period. Those used in the first test were approximately nine months old and ranged in weight from 710 to 950 grams with an average weight of 820 grams, and those in the second test, which was conducted several months later, varied from 750 to 970 grams with an average weight of 880 grams. The animals were placed in metabolism cages and were fed a scorbutogenic pelleted stock diet (commercial) supplemented with one per cent of powdered yeast. Ascorbic acid was supplied by daily intraperitoneal injections of 5 mgm. for each 100 grams of body weight. The vitamin was dissolved (15 mgm./ml.) in 0.5 per cent solution of sodium bicarbonate to partially neutralize the acidity. Each animal received the same (as the initial) dosage throughout the experiment.

Ten grams of crystalline metaphosphoric acid was placed each day in each of the bottles used for collecting the urine. This amount was usually sufficient to afford protection to the ascorbic acid excreted during a 24-hour period in a room maintained at 76 to 80°F. Some difference was observed in the quantity of metaphosphoric acid required by the urines of different animals and was attributed in part to differences in the volume of fluid collected in the bottles. In preliminary tests it was observed that some of the animals spilled water as they drank, thus diluting the metaphosphoric acid considerably. By providing drinking tubes with very small openings for animals inclined to spill the water some control was obtained of the volume of fluid collected.

Assays of the ascorbic acid excreted were made daily by both the indophenol method and the osazone method of Roe and Kuether (12) to determine the reduced and total ascorbic acid, respectively. The excretion was determined during the 9-day period previous to wounding, throughout the period of active healing (11 days in the first test and nine days in the second) and for nine days following complete healing of the wounds with the exception that osazone determinations were made for only seven days during the recovery period in the first experiment. The wounding² was effected by cutting through the skin down the center of the back for a distance of 10 cm. The cut edges were fastened together with skin clips which were removed on the ninth day after wounding, at which time healing was well advanced. Precautions were taken to keep the animals well protected from draughts and also to maintain their room at slightly higher temperature than usual during the first 24-hour period after wounding. The food and water intake was slightly less than usual during this period and

¹ Animals from family No. 13 were used. This strain was developed by the Genetics Section of the Bureau of Animal Industry of the U. S. Dept. of Agriculture. Later, the line was continued by the Genetics Section of the National Cancer Institute, from which the animals here used were obtained.

² The writer is indebted to Dr. Thelma B. Dunn of the National Cancer Institute for the surgical operations.

became normal by the second day. None of the pigs showed delayed healing due to infection.

EXPERIMENTAL OBSERVATIONS

After preliminary experiments in which the necessary methods were developed, two tests each with eight animals were conducted, the results of which corresponded fairly well within the limits of error of the method. The results of the two tests with both methods are shown in figures 1 and 2. The data indicate a real change in excretion during the healing period. The rather wide deviations

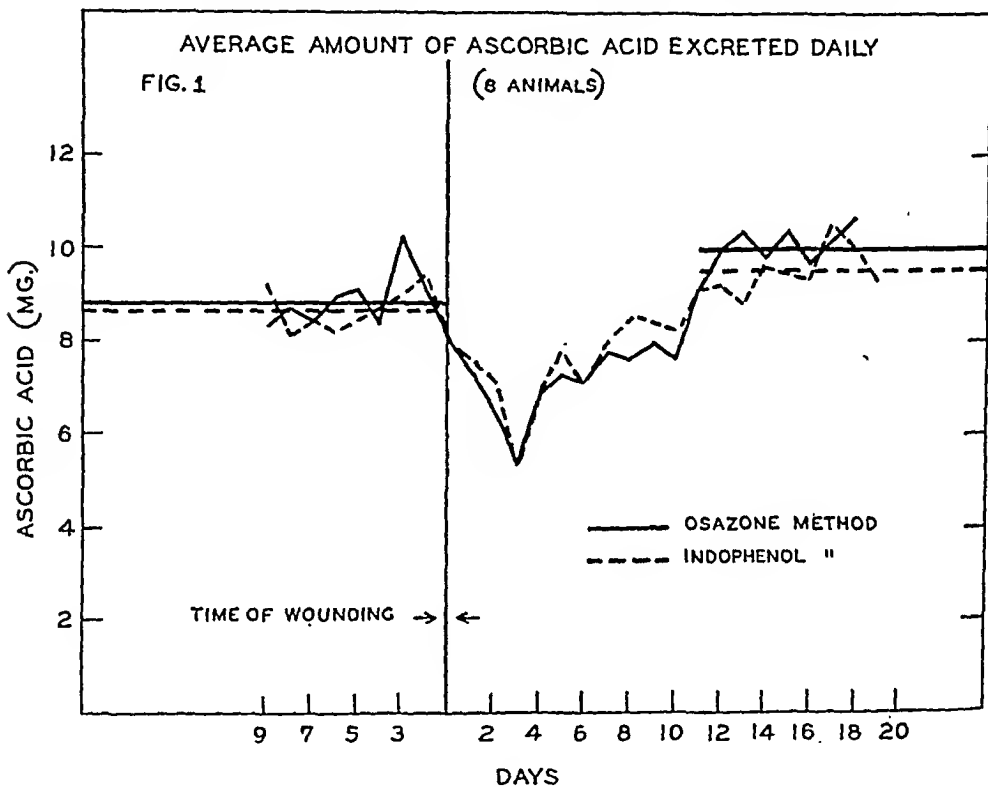


FIG. 1

occurring throughout the three experimental periods are caused in part by differences in weight and dosage of the animals and in part by individual differences³ in the average daily excretion. The horizontal lines at the left in the figures represent the average excretion values during the prewounding period and those at the right the average values during the period after healing was apparently complete. The differences in results by the two methods of assay were not great.

³ In other studies (13) it has been found that guinea pigs excrete (excess) intraperitoneally injected ascorbic acid into the digestive tract where it is fairly rapidly destroyed. There are individual variations in the rapidity of this process, the differences probably resulting from variations both in the rate of excretion into the digestive tract and in its subsequent destruction. This variability is reflected in the blood concentration and consequently would presumably influence the amount removed by the kidneys.

The generally higher values obtained with the osazone method, shown particularly in figure 2, are presumably due to the presence of small amounts of dehydroascorbic acid. The low value obtained with the osazone method on the fourth day previous to wounding in the second test (fig. 2) is probably a result of an analytical error, the cause for which is not known.

After the wounding there was a drop in the excretion values in both experiments. The most striking feature of the results is the definite fall by the third day with the values remaining low for several days. In both tests all of the animals but one showed this depression in excretion after wounding, with a

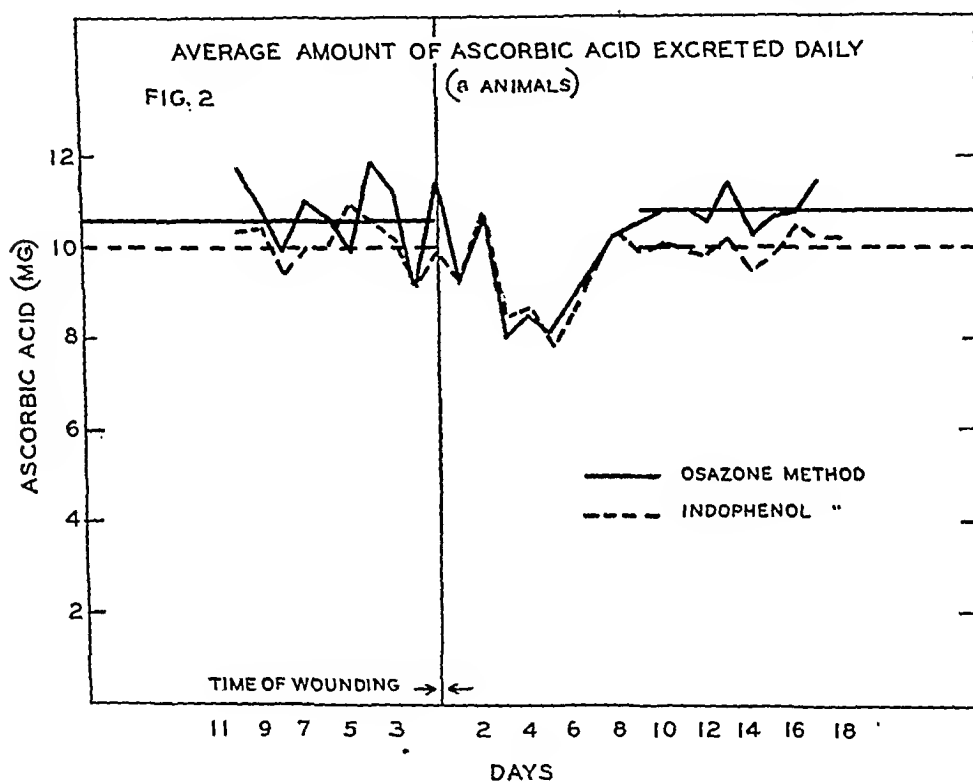


FIG. 2

return to normal values during the recovery period. The quantitative data for the total excretion values per animal during the three experimental periods are shown in tables 1 and 2. The general level of excretion during the prewounding period in the first test (table 1) was somewhat lower than is generally expected under these experimental conditions. The return to normal excretion values occurred by the eleventh or twelfth days in the first test and by the ninth day in the second. This difference in time was correlated with an apparent difference in the rate of wound healing. The animals in the second test, which required the shorter time, had in most cases, a more rapid rate of healing. During the period of active healing there was a decrease in the total amount of ascorbic acid normally excreted of 16.5 mgm. or 18.6 per cent per individual animal in the first test and in the second test a decrease of 12.4 mgm. or 12.9 per cent.

The averages for the standard deviations in excretion per individual were slightly higher during the healing than during the prewounding period. The

TABLE 1. TOTAL ASCORBIC ACID EXCRETED PER ANIMAL (EXPT. 1) DURING 7 TO 10-DAY PREWOUNDING, HEALING AND RECOVERY PERIODS
(Osazone Method)

GUINEA PIG NUMBER	PREWOUNDING 9 DAYS	HEALING 10 DAYS	RECOVERY 7 DAYS
	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>
24	78.0	52.1	54.6
26	67.5	49.8	66.6
28	74.3	62.0	70.1
29	58.6	52.4	61.5
31	91.0	110.4	83.6
32	119.5	112.1	92.8
33	63.5	60.2	55.4
35	84.9	77.0	74.5
Total.....	637.3	576.0	559.1
	708.1*	-15.3 mgm. -18.6 %	798.7† +11.3 mgm. +12.8 %

* Excretion for 10 days (calculated).

† Excretion for 10 days (calculated).

TABLE 2. TOTAL ASCORBIC ACID (MG.) EXCRETED PER ANIMAL (EXPT. 2) DURING THE 9-DAY PREWOUNDING, HEALING AND RECOVERY PERIODS
(Osazone Method)

GUINEA PIG NUMBER	PREWOUNDING	HEALING	RECOVERY
25	82.9	86.0	83.4
26	95.4	69.0	82.4
27	81.2	67.1	81.5
28	112.4	103.0	132.0
29	92.2	77.9	95.9
32	118.8	107.8	124.8
33	86.3	74.5	91.9
35	96.5	81.4	88.6
Total.....	765.7	666.7	780.5
		-12.9 % -12.4 mgm.*	+1.9 %

* Per animal.

average values for the prewounding, healing and recovery periods, respectively, were 0.91, 1.01 and 0.74 mgm. in the first test and 0.85, 1.00 and 0.98 mgm. in the second (tables 3 and 4). If a four consecutive-day interval in the middle of each

TABLE 3. AVERAGE AMOUNTS OF ASCORBIC ACID EXCRETED DAILY DURING THE PREWOUNDING, HEALING AND RECOVERY PERIODS (EXPT. 1)
(Osazone Method)

PREWOUNDING		HEALING		RECOVERY	
Days before wounding	Mean	Days after wounding	Mean	Days after wounding	Mean
	mgm.		mgm.		mgm.
1	8.10 \pm 0.70	1	7.41 \pm 0.99	11	9.06 \pm 0.95
2	9.26 \pm 0.82	2	6.51 \pm 1.02	12	9.96 \pm 0.68
3	10.32 \pm 0.91	3	5.49 \pm 1.48	13	10.38 \pm 0.32
4	8.42 \pm 1.07	4	7.40 \pm 1.12	14	9.76 \pm 0.81
5	9.13 \pm 0.96	5	7.70 \pm 1.13	15	10.39 \pm 0.76
6	8.96 \pm 0.66	6	7.17 \pm 1.02	16	9.68 \pm 0.75
7	8.42 \pm 1.08	7	7.80 \pm 0.55	17	
8	8.77 \pm 0.97	8	7.65 \pm 1.14	18	10.65 \pm 0.94
9	8.31 \pm 0.98	9	7.99 \pm 0.78		
		10	7.68 \pm 0.90		
Mean	8.85 \pm 0.91		7.28 \pm 1.01		9.98 \pm 0.74

Mean for comparison periods: prewounding, 9.21 \pm 0.90; healing, 6.94 \pm 1.19; recovery, 10.05 \pm 0.66.

* -24.6%.

† +9.1% comparison period.

TABLE 4. AVERAGE AMOUNTS OF ASCORBIC ACID EXCRETED PER ANIMAL DAILY DURING THE PREWOUNDING, HEALING AND RECOVERY PERIODS (EXPT. 2)
(Osazone Method)

PREWOUNDING		HEALING		RECOVERY	
Days before wounding	Mean	Days after wounding	Mean	Days after wounding	Mean
	mgm.		mgm.		mgm.
1	11.33 \pm 0.65	1	9.13 \pm 0.95	10	10.76 \pm 0.54
2	9.17 \pm 0.77	2	10.61 \pm 0.67	11	10.81 \pm 1.20
3	11.29 \pm 0.89	3	7.99 \pm 0.93	12	10.48 \pm 1.13
4	11.83 \pm 0.66	4	8.52 \pm 0.98	13	11.38 \pm 1.19
5	9.79 \pm 0.35	5	8.10 \pm 0.85	14	10.18 \pm 1.00
6	10.62 \pm 0.82	6	8.78 \pm 0.88	15	10.65 \pm 1.13
7	11.00 \pm 1.42	7	9.46 \pm 1.01	16	10.80 \pm 1.06
8	9.82 \pm 1.03	8	10.23 \pm 1.63	17	11.45 \pm 0.79
9	10.85 \pm 1.05	9	10.51 \pm 1.08	18	11.04 \pm 0.75
Mean	10.63 \pm 0.85		9.26 \pm 1.00		10.84 \pm 0.98

Mean for comparison periods: prewounding, 10.88 \pm 0.68; healing, 8.35 \pm 0.91; recovery, 10.67 \pm 1.11.

* -23.2%.

† +1.9% comparison period.

of the three experimental periods is selected for purposes of comparison, it is found that the average excretion during the healing period was 24.6 per cent

lower than that of the prewounding period in the first experiment and 23.2 per cent lower in the second. During the recovery period there was a return to values slightly higher (9.1 per cent and 1.9 per cent in the first and second tests, respectively) than in the comparable prewounding period.

Because of the necessity of collecting the urine in metaphosphoric acid there was no opportunity to follow any possible changes in the urinary pH values. The pH values of the urines of these animals determined shortly before starting the present experiments varied from 7.95 to 8.50. Because of the alkaline reaction some oxidation of the vitamin probably occurs during the period in which the urine is retained in the bladder (14), even though the oxygen tension is doubtless low. At these high alkalinities destruction of dehydroascorbic acid doubtless occurs rapidly, which would account for the low values found for dehydroascorbic acid (difference between total and reduced).

DISCUSSION

The feature of special interest in the results of these experiments is that they show that the period of diminished excretion of the vitamin in the urine coincides roughly with the known period of collagen formation.

Hunt (5) showed that with a sufficient supply of ascorbic acid a mature vascular scar is built up in wounded guinea pigs within 14 days. Hartzell and Stone (6) also found that the strength of healing wounds in the abdominal walls of guinea pigs adequately supplied with ascorbic acid increased sharply after six days and reached the strength of the intact abdominal wall by the 14th day. Mazoue (15) had previously studied the rate of production of collagen fibers around a mass of Kieselguhr injected intraperitoneally into guinea pigs and demonstrated a direct relation between the dosage of ascorbic acid and the time of the first appearance of fibroblasts and fibers. The time of greatest apparent cellular development occurred from around the fourth to the sixth days. The types of cells invading and developing in the wounded area varied at different stages, the polymorphonuclears, which were most conspicuous on the first and second days were less numerous by the fourth day. Cells of connective tissue type were noticeable on the second day and fibrils of the fibroglia fibril type had by this time formed a net surrounding the mass of Kieselguhr. Connective tissue cells predominated by the fourth day and showed a high degree of activity. At this time fibrils of precollagen were very numerous and more collagen fibers could also be seen. By the sixth day the fibers were much more numerous, they were thickening and uniting into bundles, encapsulating the mass of Kieselguhr. By the eighth and tenth days the mass was completely encapsulated and was traversed throughout by fibers. By this stage all of the precollagen fibers had been changed to collagen. On the 13th day the process of fibrosis was observed to be continuing until it formed a complete layer about the entire mass of Kieselguhr.

Although there is a rough correlation between the periods of increased ascorbic acid metabolism and of collagen production during wound healing, actually there appears to be a slight time difference in the maxima for the two processes.

The period of maximal disappearance of the vitamin from the urine in the present tests extended from the second to the sixth day, thus preceding slightly the known period of most active fiber production. Hence it would appear to coincide somewhat more closely with the period of maximum connective tissue cell activity.

The amounts of the vitamin which were found in scar tissue of guinea pigs by Bartlett, Jones, and Ryan (8) namely, 6.53 mgm. per 100 grams of scar tissue, could not account for the urinary output decrements found during wound healing (15.3 and 12.4 mgm.) in the present tests unless some utilization or destruction of the vitamin also occurs.

There is a discrepancy between the present results and those of Andrae and Browne (9) as to the time at which greatest retention of the vitamin occurs. They found the greatest retention immediately after injury whereas in the present tests it occurred three to six days later. The discrepancy may be explained by the difference in type of subjects, but it seems probable that it may be found, at least in part, in a different direction. In their subjects with accidental injuries there probably was a much greater relative amount of actual tissue destruction than in the clean-cut skin wounds of the present tests. Presumably, the greater the amount of cellular damage, the greater may be the actual destruction of the vitamin, a condition which may account for the greater retention immediately after injury. Later, as the cellular elements migrate into the wounded area and become active, they, or at least certain types of these cells, may (1) very actively absorb the vitamin and (2) may possibly utilize it in anabolic processes associated with healing. Evidence lending indirect support to these suggestions is to be found in the ratio of dehydro to reduced ascorbic acid in the urine. Muntoni (16) reported that after surgical operations the ratio of dehydro to reduced ascorbic acid increased. Andrae and Browne (9, 10) found also that immediately after injury the ratio increased but that during convalescence the ratio was the same as in the normal condition. The fairly large amounts of dehydro ascorbic acid formed immediately after injury may be interpreted as indicating destruction of the vitamin catabolically and the small amount or none formed during convalescence as indicating little or no destruction. The formation of little or no dehydroascorbic acid as revealed in their studies during healing suggests the possibility that the retention characteristic of this period may be a consequence of anabolic processes. In the present tests there is some indication that less rather than more dehydro ascorbic acid is formed in the healing period than in the prewounding and recovery periods. This is shown particularly in figure 2 in which the curves obtained by the two methods are divergent during the prewounding and recovery periods but follow each other rather closely during the healing period. Although it is possible that the apparent decrease in dehydroascorbic acid during healing is fortuitous, the suggestion is nevertheless advanced that since a more rapid catabolism of protein is a characteristic accompaniment of injury (17), presumably the urine at this time may have a somewhat lower pH and as a consequence less of the vitamin is changed into the dehydroform.

SUMMARY

Experiments were conducted to determine if ascorbic acid is used up in the healing of skin wounds in guinea pigs. Incisions 10 cm. long were made down the centers of the backs of adult animals. Daily intraperitoneal injections of the same amounts of ascorbic acid were continued through a nine-day pre-wounding period, through the period of active healing (9 to 11 days), and for 7 to 9 days following closure of the wounds. Daily determinations of the ascorbic acid excreted in the urine were made by both the osazone and indophenol titration methods.

In each of the two tests conducted with eight animals each, it was found that a depression in the urinary excretion of ascorbic acid occurred during the period of most active healing by both methods of assay following which there was a return to the prewounding values. The greatest depression occurred from the second to the sixth days. During the nine-day period of active healing there was a decrease of 15.3 mgm. or 18.6 per cent of the total amount normally excreted in one test and of 12.4 mgm. or 12.9 per cent in the other. The difference between the output of a four consecutive-day period at the time of lowest excretion with a comparable prewounding period was 25 and 23 per cent, respectively, in the two experiments.

The results suggest that less dehydroascorbic acid is produced in the guinea pig during the period of active healing than in the prewounding and recovery periods.

The period of greatest metabolism of ascorbic acid preceded slightly the known period of most active fiber formation, hence it appears to parallel somewhat more closely the known period of maximal connective tissue cell activity.

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ROENTGENOGRAPHIC STUDY OF MOTILITY OF GASTRO-INTESTINAL TRACT OF THE GUINEA PIG

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Received for publication December 24, 1948

Investigations dealing with the rate of movement of the contents through the different regions of the gastro-intestinal tract have been made with several types of laboratory animals. Magnus (1) studied the progress of digestion in cats and dogs starved for 24 hours using the roentgenographic technic developed by Cannon (2, 3). He fed cats each five grams of bismuth subnitrate in 25 ml. of potato brei, and dogs were given 7.5 grams of bismuth subnitrate mixed with 50 ml. of broken dog biscuit moistened with water. In similar studies with rats Menville, Ané and Blackberg (4) fasted the animals for 48 hours, withheld water 24 hours before feeding a meal composed of 10 grams of BaSO_4 and 10 ml. of buttermilk. Gershon-Cohen, Shay and Fels (5) conducted similar investigations on rats and found considerably more rapid movement of the contents of the digestive tract, which may possibly be explained in part by differences in experimental procedure. Their animals were deprived of food and water only 18 hours prior to the roentgenographical studies. The test meal consisted of three ml. of a water suspension of barium sulphate put directly into the stomach by a stomach tube. The results of these various studies together with one on human subjects (6) are summarized in table 1.

No reports have been found of investigations dealing with the rate of movement of the contents of the gastro-intestinal tract of guinea pigs. For another study, initiated to investigate the disappearance from guinea pigs of a relatively large proportion of the ascorbic acid injected intraperitoneally, such information was found to be essential.

Twenty-seven roentgenographic studies using tests meals of a water-barium sulphate mixture were accordingly made on 17 adult animals of an inbred strain maintained on a commercial pelleted diet supplemented with one per cent of dried powdered yeast. Ascorbic acid was injected intraperitoneally (daily) in the proportion of five mgm. per 100 gram of body weight. A 0.5 per cent solution of sodium bicarbonate was used as a solvent for the vitamin (15 mgm./ml.). The animals were kept on this regime for four weeks or longer before making the studies. In some of the tests the food was removed from the cages the night before the barium was administered; in others it was removed at 4 A.M. the day of the tests. At 8 A.M. five ml. of a creamy mixture of 10 grams of barium sulphate in 20 ml. of water was introduced into the stomach by stomach tube. Roentgenograms¹ were taken immediately and after 1, 2, 3, 4, 6, 8, 10 and 24 hours. After the fourth hour the food was replaced in the cages.

¹ $\frac{1}{16}$ sec., 30 milliamps, 62 kilovolts, 30" distance.

TABLE 1. RATE OF MOVEMENT OF CONTENTS THROUGH GASTROINTESTINAL TRACT IN DIFFERENT ANIMALS

ANIMAL	STOMACH: EMPTYING TIME	SMALL INTESTINE			CECUM		LARGE INTESTINE: EMPTYING TIME	INVESTIGATORS
		First appearance	Maxi- mum filling	Emptying time	First appearance	Empty- ing time		
	<i>hr.</i>	<i>min.</i>	<i>hr.</i>	<i>hr.</i>	<i>hr.</i>	<i>hr.</i>	<i>hr.</i>	
Cat.....	3	15	2	8-9	2	—	—	Magnus (1)
Dog.....	2.5	15	2	6-7	about 2	—	—	Magnus (1)
Rat.....	6 hrs. 14 min.	—	—	10 hrs. 26 min.	3 hrs. 37 min.	—	65	Menville, Ané and Black- berg (4)
Rat.....	1-3	—	—	—	3-5	—	24	Gershon- Cohen, Shay & Fels (5)
Man.....	4.6	25	—	6.5	3.3	17.3	25.5	Zehbe (6)
Guinea pig..	1+	less than 1 hr. Ex- act time not det'd	1	2	less than 1	8-10	24- to 24+	Reid and White (this paper)

TABLE 2. LOCATION OF BARIUM MEAL IN THE DIGESTIVE TRACT OF GUINEA PIGS AT SUCCESSIVE PERIODS. TWENTY-SEVEN STUDIES ON 17 ANIMALS

ORGANS	HOURS AFTER ADMINISTRATION OF BARIUM SULPHATE							
	1	2	3	4	6	8	10	24
Stomach	$\frac{1}{2}$ to $\frac{3}{4}$ out	Almost out	—	—	—	—	—	—
Small in- testine	through- out en- tire length	Almost out	all out except trace	—	—	—	—	—
Cecum	entering in 6 of 27	$\frac{1}{2}$ to $\frac{4}{5}$ of total in	$\frac{1}{2}$ to $\frac{3}{4}$ in	$\frac{1}{2}$ to $\frac{3}{4}$ in	$\frac{1}{2}$ to $\frac{1}{2}$ in	$\frac{1}{2}$ to trace in	all out or trace	—
Large in- testine	—	Enter- ing	small am- ount to $\frac{1}{3}$ in	$\frac{1}{4}$ to $\frac{1}{2}$ in	$\frac{1}{2}$ to $\frac{4}{5}$ in	feces es- caping in 5	feces es- caping in 9	all out in 16. Small am- ount in 6. Trace in 5

OBSERVATIONS

At the end of the first hour from a third to a half of the barium was still in the stomach, the remainder being distributed throughout the entire length of the small intestine. By the end of two hours most of the barium had passed into the

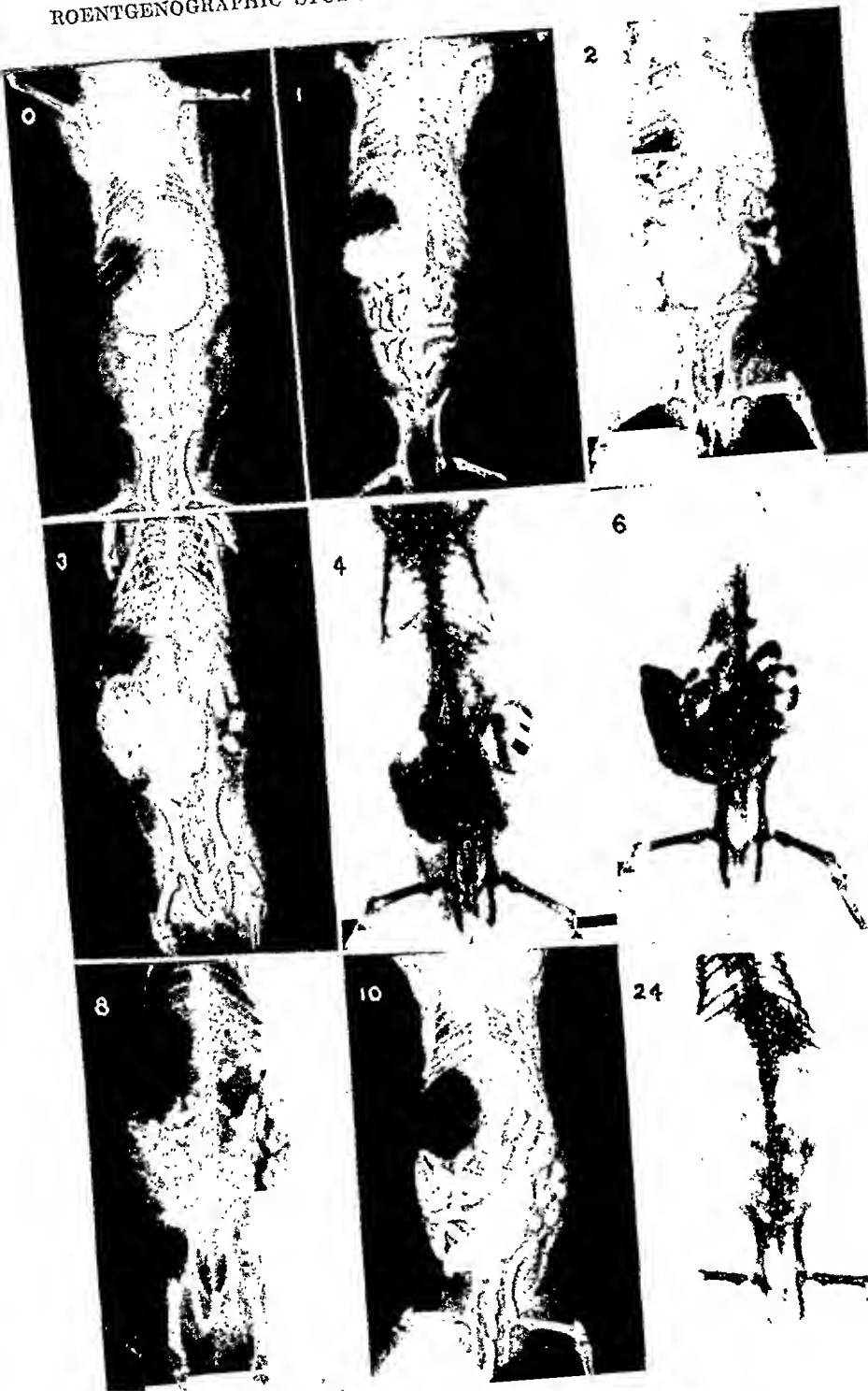


FIG. 1. LOCATION OF BARIUM AFTER DIFFERENT INTERVALS OF TIME. Figures represent number of hours after administration. The photographs do not represent the gastro-intestinal tract in any one individual but rather illustrate the average position of the barium for each time interval as near as the average could be determined.

cecum and in most of the animals it could be detected entering the large intestine. Only small amounts were left in the stomach and small intestine in most of the

animals. Some of the barium remained in the cecum at least five hours and in many cases longer. By the eighth hour it appeared to be almost all out of the cecum. After eight hours, feces containing barium were escaping in five of the 27 animals. After 24 hours the barium had all been excreted in 16 of the 27 animals, there was a trace left in the large intestine in five of them and small amounts in six. No definite differences were observed in the rate of movement of the contents of the gastro-intestinal tract between the animals deprived of food over night and those without it for only four hours previous to administering the barium.

The results of the above studies are summarized in table 2 and typical roentgenograms are shown in figure 1.

DISCUSSION

The most interesting feature of these results is the rapid progress of the barium through the stomach and small intestine of the guinea pig and the marked retardation in the cecum. The passage through the stomach is faster than that observed in the cat (1), dog (1) and man (6) and the progress through the small intestine is much more rapid than in these other animals. In general, the movement is more like that found in the rat (5), though there is a definite difference in the time required for passage through the small intestine. The first appearance of the barium was observed in the cecum of the rat in from three to five hours, whereas in the guinea pig it entered within the first hour and by the end of the second hour all but a small amount had passed in.

As to whether barium may induce more or less rapid propulsion than would normal food stuffs no definite answer can be given at the present time. Judging by the results obtained under similar conditions with human subjects, however, it may be assumed that the movement is practically normal.

The chief significance in the results is to be found in the influence of time as a factor in the absorption of special types of nutrients. Since there is considerable evidence that much if not most of the absorption of vitamin C, for example, occurs through the walls of the small intestine and possibly to some extent in the stomach, the short period during which the contents are exposed to these absorbing surfaces becomes of critical importance. From these results and the data presented in a previous publication (7), it appears probable that if the daily quota of this vitamin is given in one dose or in a form not readily available for absorption there may be considerable loss if the substance is propelled forward out of the small intestine before absorption is complete.

SUMMARY

The roentgenological-barium sulfate technic commonly employed in clinical determinations of gastro-intestinal propulsion rate was applied in guinea pigs to a study of the rate of movement of contents through the different regions of the gastro-intestinal tract.

The chief feature of interest in the results is the rapid movement through the stomach and small intestine and the marked retardation in the cecum. Within

a period of two hours most of the barium had moved out of the stomach and small intestine. After 24 hours all of the barium had been eliminated in 16 of the 27 animals, traces only were left in five animals, and small amounts in six.

The significance of the results is discussed in connection with possible failure in the complete absorption of special types of nutrients such as some of the vitamins, particularly when a 24-hour quota in high dosage is administered at one time, or in a form not readily accessible for absorption.

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EFFECT OF FEEDING LIVER ON THE RATE OF REGENERATION OF THE LIVER IN PARTIALLY HEPATECTOMIZED RATS

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Received for publication January 5, 1948

This study was undertaken to ascertain whether or not the feeding of liver to partially hepatectomized rats augments the rate of regeneration of the liver.

This question was raised because we were interested in the more basic question of the nature of the stimulus or stimuli responsible for the remarkable regenerative ability of the liver. The stimulus which is responsible for the regeneration of the liver may be extrinsic or intrinsic in origin. If the stimulus is intrinsic and is not mechanical, then it should be present either in the nonregenerating liver or in the regenerating liver. If such a factor be present in the liver in either state, it should be detectable by feeding whole liver in large amounts or by injecting appropriately prepared extracts or concentrates of the liver into partially hepatectomized rats. If a favorable effect occurred on feeding liver, the possibility that the effect might be due to the quality of liver protein (1) would have to be considered. It was thought that the best approach to the answer of the basic question was to ascertain the effect of feeding a diet of liver on the rate of regeneration of the liver as compared to the feeding of a stock diet.

We have been unable to find any literature dealing directly with this question. McJunkin and Breuhaus (2) observed a great increase in mitosis in the hepatic remnant of partially hepatectomized rats which had received injection of 'mac-erated liver substance'. Marshak and Walker (3) injected various liver fractions into partially hepatectomized rats and observed an increase in mitosis when fat-free chromatin was injected. They found in addition that chromatin labeled with P32 was rapidly assimilated into the intracellular substance of the regenerating liver. This work suggests that the liver may contain some factor which stimulates regeneration.

EXPERIMENTAL

Technique of removing the liver. At operation, the method of Anderson and Higgins (5) and Brues, Drury, and Brues (4) was used. It should be mentioned that the portal vein leading to the lobes to be removed was clamped for three minutes to allow for partial exsanguination of the lobes before their pedicle was tied. The portion removed was allowed to drain of blood and was then weighed to the nearest five milligrams.

At 'necropsy'; as in the 32 rats cited below, the rat was anesthetized on the tenth to eleventh day (average, 10.7 days), the abdomen was opened, the portal vein and hepatic artery were clamped, and then after two or three minutes the vena cava was clamped and the liver removed. The liver was allowed to drain of blood, weighed wet, and then dried to constant weight in a constant tempera-

ture oven at 100° C., it having been found that further desiccation in a desiccator did not decrease the weight.

Amount of liver removed. We desired to remove approximately 70 per cent of the liver as others have done. In this connection Brues, Drury and Brues (4) found that the left lateral and the median lobes of the rat's liver constituted 68.5 per cent of the total weight of the liver. Or, in 13 rats from their colony they found that if the weight of these lobes was multiplied by the factor 1.46, the total weight of the liver could be approximated. This factor of 1.46 had a standard deviation of only ± 0.03 for the series of 13 rats they used. This, of course, served as a basis for the amount of liver regenerated after partial hepatectomy. They found that this method had a smaller coefficient of variability than when the formula for body-weight to liver-weight relationships was used (5).

To establish for our colony of rats the validity of the method of Brues *et al.* (4) for estimating the total weight of the liver at the time of operation, we removed the left lateral and median lobes of the liver and weighed them; then we removed and weighed the remainder of the liver (left central, right, and caudate lobes).

TABLE 1

RATS	AVR. BODY WT. AT OP. (GRAMS)	AVR. WT. OF MED. AND LEFT-LAT. LOBES REMOVED AT OP.	AVR. WT. OF RIGHT AND CAUDAL LOBES REMAINING	AVR. WT. OF TOTAL LIVER
32	180.4 \pm 4.3 ¹	1.798 gm.	0.824 gm.	2.628 gm.

Thus, the ratio of $\frac{\text{med. and left lat.}}{\text{Total}} = \frac{1.798}{2.623} = 68.56\% \pm 0.27 \text{ (P.E.)}^1$

Range of percent of liver removed = 64.0% to 71.3%.

¹ Probable error of the mean.

Thirty-two albino rats, which weighed from 150 to 300 grams and which were approximately equally distributed between the sexes, were used.

The averaged results are shown in table 1. The value of 68.56 per cent \pm 0.027 (P.E. of the mean) checks the percentages of Brues very precisely, the factor being 1.46 ± 0.047 (standard deviation).

The effect of feeding a diet of liver on the rate of regeneration. Prior to operation the rats were kept on the stock diet used in our laboratory and water. The diet had the following composition: P = 22%, CHO = 45%, F = 4.0%, and fiber = 5%, and was adequate to support normal group and reproduction. The composition of the pigs' liver was approximately: P = 20%, CHO = 1.0%, and F = 4.8%. The 'control group' was given the diet and water after operation. The 'liver-fed group' was given only ground pigs' liver which had been brought to a boil to stop autolysis and to retard putrefaction. The food and water were given *ad libitum*, and the rats were kept in individual cages. No pre- or post-operative withdrawal of food was practiced; neither was saline or glucose given post-operatively. Food was placed in the cage immediately after the operation.

The rats were paired according to body weight so that the average weight of the 'control group' would be approximately the same as that of the 'liver-fed

group.' The 'control' and 'treated' groups were treated alike as regards operative technique.

Since it has been reported that starvation retards the regeneration of the rat's liver (6, 4, 7), and since Higgins and Anderson (5) observed that a control laparotomy would cause the loss of about 12 to 15 per cent of the body weight

TABLE 2. DATA ON PARTIALLY-HEPATECTOMIZED RATS SHOWING THE EFFECT OF FEEDING PIGS' LIVER FOR TEN DAYS

PROCEDURE	NO. OF RATS	AVR. BODY WEIGHT		AVR. WT. DRY LIVER AT NECR., 10.7 DAYS POSTOP.	AVR. WT. DRY LIVER REMOVED AT OPERATION	CALCUL. AVR. DRY WT. LEFT IN AT OP.	LIVER DRY WT. INCREMENT, FROM OP. TO NECR.
		At op.	At necr.		(X 0.46 =)		
Treated rats (fed liver)	22	217.9 ±7.2 ¹	213.6 ±7.0 ¹	2.276	1.356	0.624	1.652 ±0.06 ¹
Control rats	29	215.3 ±5.6 ¹	208.2 ±5.4 ¹	1.782	1.386	0.638	1.144 ±0.03 ¹

$$\text{Diff.} = 0.508 \\ \pm 0.09^1$$

¹ Probable error of the mean.

$$\text{Formulae: P.E. mean} = 0.6745 \frac{\sqrt{\frac{\sum(\delta)^2}{n}}}{\sqrt{n-1}}$$

where $\sqrt{n-1}$ is applicable for $n > 20$, or < 30 .

$$\text{P.E. diff.} = \sqrt{(\text{P.E.}_{m1})^2 + (\text{P.E.}_{m2})^2} = \pm 0.0716$$

$$\text{Critical Ratio (C.R.)} = \frac{\text{diff.}}{\text{P.E. diff.}} = 7.1$$

TABLE 3

PROCEDURE	NO. OF RATS	AVR. BODY WEIGHT		AVR. DRY WT. INCREMENT OF LIVER RESTORED	SIGMA	P.E.
		At op.	At necr.			
Controls	16	202.8	200.5	1.367 gm.	0.312	0.056
Wilson's conc. liver extr.	13	218.0	215.0	1.335 ¹	0.434	0.088
Thiamine HCl	10	206.8	210.2	1.374 ¹	0.309	0.077

¹ If these increments are compared to the control of 1.144 obtained on 29 rats in table 2, the difference is not significant.

during 10 days, we believed it was necessary to control this factor. With this idea in mind the data from no rat have been used in making comparisons between the 'control' and 'liver-fed' groups if at 10.7 days more than 15 grams of weight had been lost.

The average results are shown in table 2. It is to be noted that the average body weight of the 29 control and 22 liver-fed rats was approximately the same at the time of the operation and at necropsy. However, the liver-fed rats

regenerated 0.5 gram more dry liver substance than the control rats. This difference is statistically significant (C.R. = 7.1). The same is true of wet liver substance.

The effects of thiamine and a liver extract on the rate of regeneration. The significance of the foregoing observation is enhanced by the lack of effect of thiamine and of a liver extract used in the treatment of pernicious anemia (table 3).

In this experiment the rats were treated in exactly the same way as the foregoing group, except that all the rats received the stock diet, and that out of the 39 rats used, 16 served as a group of controls, 10 were given 200 micrograms of thiamine hydrochloride daily subcutaneously, and 13 were given subcutaneously 5 U.S.P. units of a concentrated liver extract which is used for the treatment of pernicious anemia (Wilson Laboratories, Chicago).

The ratio of dry-liver weight to wet-liver weight in the rats receiving the stock diet and those receiving pigs' liver. The significance of the favorable effect of pigs' liver

TABLE 4. RATIOS OF DRY LIVER WEIGHT TO WET LIVER WEIGHT

	RATS ON CONTROL DIET		RATS FED PIGS' LIVER	
	At operation	At necropsy	At operation	At necropsy
Wet wt.	$\frac{129.6}{26} = 4.985$	$\frac{181.3}{28} = 6.474$	$\frac{94.18}{20} = 4.709$	$\frac{148.49}{20} = 7.425$
Dry wt.	$\frac{40.303}{29} = 1.386$	$\frac{51.65}{29} = 1.782$	$\frac{29.83}{22} = 1.356$	$\frac{50.068}{22} = 2.276$
Ratios dry/wet.	$\frac{1.386}{4.985} = 0.279$	$\frac{1.782}{6.474} = 0.275$	$\frac{1.356}{4.709} = 0.282$	$\frac{2.376}{7.425} = 0.306$

is also supported by the data on the greater amount of dry substance accumulated by the regenerating liver cells of the rats fed pigs' liver (see table 4).

DISCUSSION

It is clear from the results of this experiment that a diet consisting solely of liver increases the rate of regeneration of the liver of a partially hepatectomized rat as compared to an ordinary stock diet which is adequate for the general nutrition of the body. The results also show that the active principle in the liver is neither thiamine nor the antipernicious anemia factor both of which are found in pigs' liver.

More definitive experiments will have to be performed to ascertain whether the effect of a liver diet on regeneration is due to a high protein intake, hepatic protein having a favorable amino acid composition or a growth-promoting substance other than thiamine and the antipernicious anemia factor.

CONCLUSION

Liver tissue, either because it contains a high quality protein or some as yet undetermined growth-promoting factor other than thiamine or the antipernicious

anemia factor, facilitates the rate of regeneration of the liver in the partially hepatectomized rat.

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METABOLIC RECOVERY RATES FROM EXERCISE AFTER ALTERATION OF ALKALINE RESERVE¹

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Received for publication January 6, 1948

Previous experiments have indicated that the limiting factors in respiratory gas exchange during recovery from exercise were probably circulatory in nature (1). This suggested the possibility that the CO₂ carrying capacity of the blood (or exchange capacities in the lungs and tissues) may be involved in the rate of CO₂ elimination. Accordingly some of the relationships between alkaline reserve and recovery rates from exercise have been studied.

A standard step-up exercise was used consisting of 20 steps per minute onto a nine-inch platform for three minutes. The methods for measuring CO₂ elimination and O₂ consumption after exercise were the same as those described previously (1). In brief the method consisted of continual and simultaneous measurements of CO₂ and O₂ in the expired air with thermal conductivity analyzers. Knowing the percentages of the gases and corresponding minute ventilations, the recovery curves for O₂ consumption and CO₂ elimination could be calculated.

The specific measurements obtained that describe the recovery period are: 1) recovery half-time constants of gas exchange, 2) metabolic gas exchange at zero recovery time and 3) O₂ debt and excess CO₂ eliminated during recovery. Half-time constants of recovery were obtained by plotting gas exchange recovery curves (above resting) semilogarithmically against time. These half-time constants represent rates of recovery; thus, a small or large time constant corresponds to a fast or slow rate of recovery, respectively. The metabolic gas exchange at zero recovery time was obtained by extrapolation of the exponential recovery curve to zero recovery time. This corresponds to A_0 in the general exponential recovery equation $A = A_0 e^{-kt}$ where A is the gas exchanged at any time t , and k is the rate constant. O₂ debt and excess CO₂ were obtained by integration of the exponential recovery equation, which yields the general form: cc. of gas = $A_0 K T$, where T is the half-time recovery constant in seconds and K is a conversion constant.

Control exercise tests were performed the day preceding the experimental tests. The latter tests were made 3 to 6 hours after ingestion of NaHCO₃ and 12 to 16 hours after ingestion of NH₄Cl. The majority of these tests were made on one experienced subject. No direct measurements of the alkaline reserve were made. However, it is assumed that ingestion of 20 grams of NaHCO₃ increases the

¹ The work in this paper was carried out under a contract between the University of California and the Aeromedical Laboratory, Wright Field, Ohio, AAF Contract W-33-038 AC-14290.

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alkaline reserve markedly (2). Likewise, NH_4Cl has been shown to decrease the alkaline reserve (2, 3).

Typical gas exchange recovery curves after ingestion of NaHCO_3 , compared with control curves, are shown in figure 1. Since there are intra-individual variations in recovery rates, in addition to experimental errors, it was necessary

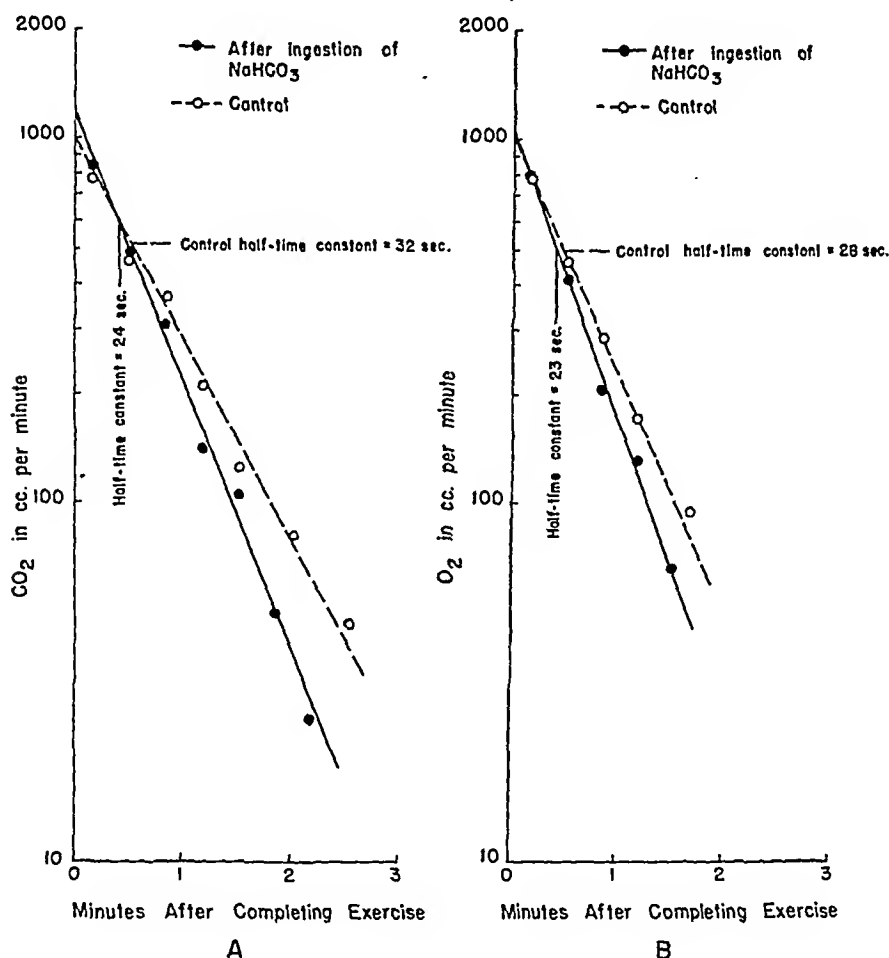


Fig. 1. SEMI-LOGARITHMIC plots of CO_2 elimination and O_2 consumption (above resting values) after ingestion of NaHCO_3 compared to control recovery curves. (Subject, W. E. B.) A. Effect of ingestion of NaHCO_3 on rate of recovery from moderate exercise as measured by CO_2 elimination. B. Effect of ingestion of NaHCO_3 on rate of recovery as measured by O_2 consumption.

to carry out a number of tests and make a statistical analysis of the data. Summarized in table 1 are results obtained on recovery CO_2 elimination following the increase in alkaline reserve by the ingestion of 20 grams of NaHCO_3 on five different days. A few hours after ingestion of the NaHCO_3 there was an average 23 per cent decrease in the time constants, which represents a 23 per cent increase in rate of recovery from exercise. Furthermore, the CO_2 elimination at zero recovery time, A_0 , was increased by 26 per cent. On the other hand there was no significant change in the total excess CO_2 eliminated during the recovery period.

In table 2 are summarized the data concerned with O_2 consumption during the recovery period. There was a significant 13 per cent decrease in the O_2 time constants, i.e., a 13 per cent increase in rate of recovery and a 9 per cent increase in O_2 consumption at zero recovery time; however, there was no significant change in the oxygen debt.

TABLE 1. EFFECT OF INGESTION OF $NaHCO_3$ ON CO_2 ELIMINATION DURING RECOVERY FROM EXERCISE
(Subject: W. E. B.)

METABOLIC MEASUREMENTS	CONTROL EXERCISE TESTS		EXERCISE TESTS AFTER INGESTION OF $NaHCO_3$		DIFFERENCE
	No. of tests	Average	No. of tests	Average	
Recovery half-time constants of CO_2 elimination (seconds)	39	31.1 ± 2.4	18	23.9 ± 2.8	23% decrease ($t = 6.4$, sig. at 1% level)
CO_2 eliminated at zero recovery time (cc./min. above resting)	39	866 ± 70	18	1090 ± 125	26% increase ($t = 4.9$, sig. at 1% level)
cc. excess CO_2 eliminated during recovery (above resting)	33	640 ± 45	14	618 ± 64	6% decrease (not significant)

TABLE 2. EFFECT OF INGESTION OF $NaHCO_3$ ON O_2 CONSUMPTION DURING RECOVERY FROM EXERCISE
(Subject: W. E. B.)

METABOLIC MEASUREMENTS	CONTROL EXERCISE TESTS		EXERCISE TESTS AFTER INGESTION OF $NaHCO_3$		DIFFERENCE
	No. of tests	Average	No. of tests	Average	
Recovery half-time constants of O_2 consumption (seconds)	34	27.3 ± 2.4	16	23.9 ± 1.1	13% decrease ($t = 4.5$, significant)
O_2 consumed at zero recovery time (cc./min. above resting)	34	970 ± 60	16	1060 ± 54	9% increase ($t = 3.4$, significant)
Oxygen debt (cc. O_2)	39	630 ± 41	18	595 ± 38	3% decrease (not significant)

Due to the unpleasant nature of the experiments these tests were made of one experienced subject. In an attempt to broaden the conclusions four additional subjects were used; two of these showed a 10 per cent decrease in their CO_2 recovery time constants after ingestion of 10-15 grams of $NaHCO_3$, and two showed no change. These results, however, are only tentative since one test was carried out per person and a statistical analysis is not possible.

In another series of tests on the experienced subject, the alkaline reserve was decreased by ingestion of 13 grams of enteric coated NH_4Cl tablets 12 to 16 hours

before the exercise tests. This apparently had no effect on CO_2 recovery rates as indicated in table 3. There may have been an effect on the O_2 consumption recovery as there was a 7 per cent decrease in the time constants of O_2 consumption after ingestion of NH_4Cl .

TABLE 3. EFFECT OF NH_4Cl INGESTION ON RECOVERY RATES FROM EXERCISE
(Subject: W. E. B.)

RECOVERY MEASUREMENTS	CONTROL EXERCISE TESTS		EXERCISE TESTS AFTER INGESTION OF NH_4Cl		DIFFERENCE
	No. of tests	Average	No. of tests	Average	
Half-time constant of CO_2 elimination (seconds)	39	31.1 ± 2.4	10	31.0 ± 2.2	1% decrease (not significant)
Half-time constant of O_2 consumption (seconds)	34	27.3 ± 2.4	10	24.2 ± 1.9	7% decrease (significant at 5% level)

DISCUSSION

Hill (4) and later Margaria *et al.* (5) distinguish two phases of recovery from an exhausting exercise. The oxygen consumption recovery curve after such an exercise is composed of an initial rapid exponential drop followed by a considerably slower exponential curve. The initial rapid phase is, according to Margaria, payment of an 'alactacid' oxygen debt and presumably represents energy for the resynthesis of phosphagen split during anaerobic muscular contraction. The slower recovery phase, or payment of the 'lactacid' oxygen debt, is concerned with oxidation of the accumulated lactic acid. Recovery from a moderate exercise is predominately or entirely repayment of the alactacid type of oxygen debt since there is no accumulation of lactic acid. In the present experiments it was decided to avoid complicating the recovery curves with two exponential phases, so the exercise chosen was of such mild intensity as to be followed only by the rapid recovery phase.

Previous evidence had indicated that the limiting factors operative during the rapid recovery phase were circulatory in nature and it was thought that alteration of alkaline reserve might conceivably influence this type of recovery, particularly as measured by the rate of elimination of carbon dioxide. As far as is known the effects of acidosis or alkalosis on the rapid recovery phase have not been studied. Dennig (6) and Margaria (5) concluded that alkalosis could improve the performance of exhaustive work by allowing greater accumulation of lactic acid, although Margaria found no effect on the velocity constants of the slower exponential recovery curve. The effect of alkalosis on the velocity constants of the rapid recovery phase was not determined.

These experiments, on one experienced subject, demonstrate that increasing the alkaline reserve by ingestion of NaHCO_3 significantly increases the rates of recovery from a mild exercise. The results lend additional support to the view that rates of metabolic gas exchange after a mild exercise are limited by circula-

tory factors. It seems probable that increased alkaline reserve results in a greater CO_2 carrying capacity of the blood which allows more rapid elimination of metabolic CO_2 from the muscles. Also, the increase in O_2 recovery rates may be related to altered CO_2 tensions which favor greater O_2 exchange capacities of the blood. Tests were carried out on a few other subjects, however at present there are insufficient data to determine the universality of these results.

The effect of alkalosis on recovery from an exhausting exercise was not tested, although, on the basis of the present results and on Margaria's results, one might expect that alkalosis would affect the velocity constants of the rapid recovery phase and would have no effect on the slower recovery curve.

Attempts to decrease recovery rates by lowering alkaline reserve were not successful; however, assuming a decreased alkaline reserve, reflected by a lowered CO_2 carrying capacity of the blood, there might have occurred a compensatory increase in the rate of blood flow, thus accounting for the slight increase in O_2 consumption recovery rates. On the other hand, there may be some question as to whether the amount of NH_4Cl used was sufficiently effective in lowering alkaline reserve.

These results suggest that intra- and inter-individual differences in recovery rates may be related in part to alkaline reserve.

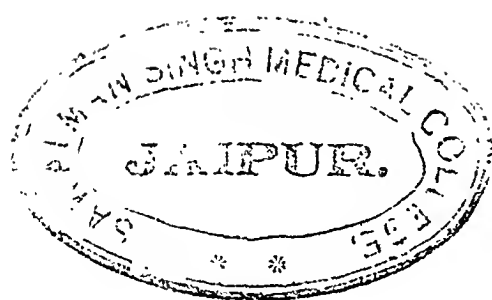
SUMMARY

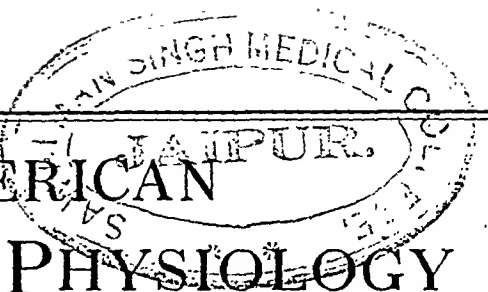
Tests carried out on one experienced subject showed that an increase of alkaline reserve by ingestion of 20 grams of NaHCO_3 brought about a 23 per cent increase of the rate of recovery from moderate exercise as measured by CO_2 elimination. There was a corresponding increase in the recovery rate as measured by O_2 consumption although to a lesser degree (13 per cent increase). Ingestion of NH_4Cl , with a presumable decrease in alkaline reserve, had no effect on the CO_2 recovery rate although a slight increase in the O_2 rate occurred. These results are interpreted as supporting the concept that the blood and circulation are limiting factors in metabolic gas exchange during recovery from a mild exercise.

The author is indebted to Miss Muriel Johnston for assistance in carrying out the experiments.

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THE AMERICAN JOURNAL OF PHYSIOLOGY

Published by
THE AMERICAN PHYSIOLOGICAL SOCIETY

VOLUME 152

MARCH 1, 1948

NUMBER 3

EFFECTIVE OSMOTIC PRESSURE OF THE PLASMA PROTEINS AND OTHER QUANTITIES ASSOCIATED WITH THE CAPILLARY CIRCULATION IN THE HINDLIMBS OF CATS AND DOGS

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Received for publication December 19, 1947

The principal factors regulating the exchange of fluid between capillaries and tissue spaces were outlined by Starling (1) following his experiments on the absorption of fluid from the connective tissue spaces. Starling's experiments, performed on perfused hindlimbs of dogs, gave rise to one of the most widely recognized hypotheses in physiology and medicine. According to the 'Starling Hypothesis', the direction and rate of fluid transfer between plasma and tissue fluids are determined by three factors; *a*) the hydrostatic pressures on each side of the capillary membranes, *b*) the protein osmotic pressures of plasma and tissue fluids acting across the capillary membranes, and *c*) the physical properties of the capillary membranes considered as mechanical filters.

Convincing quantitative evidence in support of the Starling theory was obtained by Landis (2) from direct measurements of the hydrostatic pressure and rates of fluid movement in individual capillaries of the frog's mesentery. In this preparation the rate of fluid movement across the capillary membrane is, on the average, proportional to the difference between the mean hydrostatic pressure in the capillary and the protein osmotic pressure of the plasma as measured in vitro. This behavior represents a special case of the Starling theory in which tissue pressure opposing filtration is negligible and the capillary membranes are almost completely impermeable to the plasma proteins; it implies that the protein concentration in tissue fluid immediately outside the capillary wall is too low to play a significant role in determining the osmotic balance.

No such direct measurements have been made in mammalian capillaries. Circumstantial evidence, reviewed by Landis (3), has in general lent support to the

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Starling theory, but the quantitative relations between capillary pressure, effective protein osmotic pressure and rate of fluid exchange have never been clearly demonstrated in the mammalian circulation. In the present paper we shall describe methods for measuring these variables in the isolated perfused hindlimbs of cats and dogs. Quantitative evidence will be given that the Starling theory is in fact applicable to the perfused hindlimb and with a precision rarely encountered in biological preparations.

METHODS

A. GENERAL. The hindlimbs of cats or dogs were amputated and suspended from a sensitive recording balance. They were supplied with blood from a pump-lung circulation under conditions such that the arterial perfusion pressure, the venous pressure and the protein osmotic pressure of the plasma could be independently adjusted to desired constant values. The rate of filtration of fluid from blood to tissues was recorded as the rate of gain of weight, a technique which has been employed in several previous investigations (4-6); conversely, absorption of fluid from tissues to blood was recorded as a loss of weight. The blood flow was measured and recorded continuously. From these quantities it is possible, by the methods described below, to determine the mean hydrostatic pressure in the capillaries, the effective osmotic pressure of the plasma proteins, the filtration coefficient of the capillary membranes and certain other quantities important to the fluid exchange.

B. DETAIL. 1. *Perfusion.* The technique of perfusion was similar to that described by Whittaker and Winton (7). A diagram of the perfusion circuit is shown in figure 1.

2. *Preparation of blood for perfusion.* Three hundred to 500 cc. of perfusion fluid were used for each experiment. The blood was drawn from the carotid arteries of one or more animals not more than three days prior to the experiment. For experiments with cats the blood was heparinized; with dogs the blood was usually defibrinated. When a high protein osmotic pressure was required, the plasma was separated from the cells and placed in cellophane bags in front of a fan. Loss of water by evaporation from the surface of the cellophane occurred at a rate sufficient to double the concentration of protein in about four hours. The bags were then sealed close to the liquid level and the concentrated plasma dialyzed against cold Ringer's solution (0.90% NaCl, 0.042% KCl, 0.024% CaCl₂, 0.020% NaHCO₃) until ionic equilibrium was established as indicated by the electrical conductivity. The pooled plasma concentrates from several cats were required for experiments involving high protein pressures. Low protein pressures were obtained by diluting whole blood with Ringer's solution. It was noted that the protein osmotic pressure of plasma obtained by 'bleeding out' from the carotid arteries was considerably less than normal, the average pressure being 15 mm. Hg. Presumably this was the result of dilution of the plasma with tissue fluid during the bleeding process.

The corpuscular concentration was adjusted to desired levels by the addition of red cells to the previously prepared plasma. The whole fluid was filtered

through glass wool and two layers of linen cloth before admission to the perfusion reservoir.

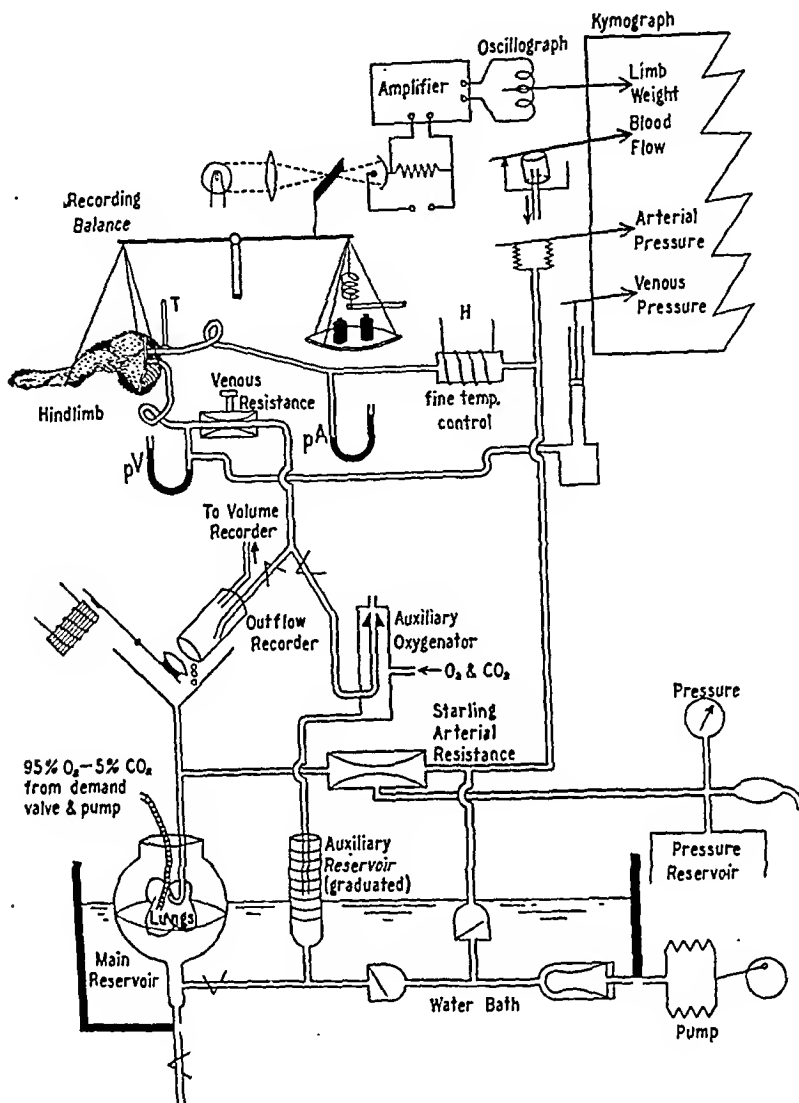


FIG. 1. DIAGRAM OF THE PERFUSION CIRCUIT AND WEIGHT-RECORDING SYSTEM. The pump-lung-hindlimb circulation is similar to that employed by Whittaker and Winton (7). The leg is mounted horizontally on the balance with the ventral surface facing upwards. The balance beam axle rotates on ball bearings. The beam is loaded with a spring which lengthens 0.21 mm. per gram; optical and electrical amplification of this displacement allow full scale deflection (50 mm.) of the recording oscillograph for a change of one gram on the balance. Temperature of blood in the arterial cannula is measured by thermometer (T) and controlled by heating coil (H).

3. *Operative procedures.* All experimental animals were anesthetized with nembutal (40 mgm. per kgm.). The right hindleg from a small dog (5 to 6 kgm.) or a large cat (2.8 to 4 kgm.) was separated from the body at the hip, all visible blood vessels being ligatured except the femoral artery and vein. Small bleeding

points were seared with a cautery. The operation required one and one-half to two hours. The femoral vein was cleared of connective tissue as far down as the saphenous branch; this was necessary to prevent compression of the vein from the weight of unsupported muscles pulling on the tissue immediately adjacent to the vein.

During this operation the lungs were removed from a second animal and the pump-lung circulation started about one-half hour before the preparation of the leg for perfusion was complete. Arterial and venous perfusion cannulae of sizes appropriate to the individual vessels were fitted to the perfusion apparatus and calibrated as described in section 4 below. The femoral artery and vein were then cut and the leg transferred to the perfusion apparatus: transfer from the natural to the artificial circulation required about two minutes.

The circulation through the leg prepared in this way was such that in the first hours of perfusion all the perfusing blood left the leg via the femoral vein. There was no detectable drainage from the lymphatics. Towards the end of each experiment, however, a small amount of blood (less than two per cent of total flow) left the leg by oozing from small vessels which had been cut during the operation. The protein content and corpuscular concentration of this blood were indistinguishable from those of venous blood in the two instances in which these quantities were compared. When dye was injected into the arterial perfusion cannula the whole leg rapidly became colored up to the cut surfaces of the skin and muscle.

4. *Pressures.* The mean arterial and venous pressures were determined with mercury and blood manometers, respectively. The manometers were connected to the perfusion circuit at some distance from the arterial and venous perfusion cannulae so that it was necessary to correct for the pressure drop across the tubing and cannulae in order to obtain the true pressure in the artery and vein at each flow rate. Since a change of 0.5 mm. Hg in mean capillary pressure produced an easily detectable change in rate of fluid movement, it was necessary to devote considerable attention to these corrections. The pressure-flow characteristics of each cannula were obtained before and after each experiment by substituting a short length of wide bore tubing for the leg and determining the pressure difference across the cannulae at various flow rates in the range used during the experiment. Pressure-flow diagrams were then constructed for each cannula and the appropriate corrections for each flow rate during the experiment derived from the smoothed curves. The true zero of each manometer was determined by extrapolating the indicated pressure-flow diagram to zero flow. All pressures are referred to the level of a horizontal plane bisecting the leg. The uncertainty in determining this level, and hence the absolute value of all hydrostatic pressures, was about ± 0.5 mm. Hg.

5. *Blood flow.* The rate of venous outflow was measured at intervals by a stopwatch and measuring cylinder; it was also recorded simultaneously by a Gaddum (8) outflow recorder.

6. *Weight.* The center of the balance beam was fitted with a steel axle which rotated on ball bearings. One arm of the balance was loaded with a helical spring

which lengthened 0.21 mm. per gram. The main weight of the limb was balanced with calibrating weights, as indicated in figure 1, and *changes* in weight were recorded from the small off-balance excursions of the spring-loaded arm. The excursions were amplified approximately 100-fold and were recorded on the kymograph as follows: one arm of the balance was provided with a shutter which interrupted light focussed on a photo tube (Cetron CE-2). The output of the photo tube was led through an impedance changer (6SJ7) and transconductance stage (6AG7) to a Weston Model 30 moving coil relay modified as described by Winton (9) to operate as a recording oscillograph. Stabilizing devices were employed for all voltage supplies so that at equilibrium temperatures the overall drift in the recording system corresponded to a change of less than two mgm. per minute on the balance. The sensitivity of the system was controlled electrically and was generally operated so that one gram on the balance produced a deflection of 20 to 30 mm. on the kymograph. The deflection of the recorder was linearly related to the weight and was calibrated at intervals during the experiment by adding gram weights to the balance. The rubber tubing which connected the perfusion cannulae on the balance to the perfusion system did not interfere with the measurements, owing to the small displacement of the balance (0.4 mm.) required for full scale deflection of the recorder. The leg itself lay horizontally with the flat dorsal surface supported by a malleable coarse mesh wire screen which was attached to the balance arm by means of three chains of adjustable length.

7. *Temperature control.* Blood in the arterial cannula was maintained at 37.5°C. at all flows by means of a heater coil (H, fig. 1) operated from a manually controlled variable transformer.

8. *pH.* The pH of plasma or of protein solutions was measured with a glass electrode (Beckman).

9. *Protein osmotic pressure.* In vitro measurements of protein osmotic pressure were made with a Hepp osmometer (10) as modified by Brown (11). In our hands multiple determinations of osmotic pressure performed on samples of the same protein concentration agreed within a standard deviation of ± 0.7 mm. Hg. The absolute values of protein osmotic pressure found at any given protein concentration and pH agreed with the values calculated from the data of Scatchard, Batchelder and Brown (12, 13) as indicated in table 1. Calculation of the molecular weight of albumin from the observed osmotic pressures of bovine albumin lead to a value of 69,000, which is in close agreement with values obtained by independent methods (14). The measurements were made against 0.15 molar NaCl at room temperature with the plasma or protein samples in equilibrium with ambient air. Since the samples in vivo were equilibrated with a $p\text{CO}_2$ of 40-45 mm. Hg, the pH was higher in the osmometer than in the capillary circulation. The effects of a change in pH from 7.2 to 7.7 may be predicted from the equation of table 1; they were too small to be detected with our apparatus and are not considered in the final figures. However, all data were corrected to 37°C., the observed values being multiplied by the factor $310 \div (273 + t^\circ\text{C.})$. The actual temperature in the capillaries during the perfusion was less than 37°C. by

an amount which varied with the rate of blood flow; an error not exceeding +2 per cent may arise from neglect of this factor.

EXPERIMENTAL

1. *The effects of arterial pressure on the limb weight.* Figure 2 shows the effects of variations in arterial pressure on the limb weight and blood flow, the venous pressure and the protein pressure remaining constant. The arterial pressure was initially set to maintain the limb at constant weight. A sudden rise from 91 to 110 mm. Hg produced an initial increment of weight followed by a slow steady gain in weight. Presumably the initial rise is a result of an increased vascular

TABLE 1. COMPARISON OF PROTEIN CONCENTRATION WITH PROTEIN OSMOTIC PRESSURE

SAMPLE	PROTEIN CONCENTRATION	PROTEIN OSMOTIC PRESSURE, MM. Hg	
		Observed	² Calculated from protein concentration
	grams/100 cc.		
1. Cat plasma.....	4.8	13.6 ± .7	14.0
2. Bovine albumin pH 5.7.....	4.1	14.7 ± .2	14.3
3. Cat plasma.....	5.2	16.2 ± .6	16.0
4. Cat plasma.....	6.0	20.8 ± .8	20.6
5. Cat plasma + bovine albumin.....	8.7	45.3 ± .8	50.1

¹ Calculated from protein nitrogen with exception of sample 2 which was estimated from the density. We are indebted to Dr. Hegsted and members of the Department of Nutrition, Harvard Medical School, for performing the Kjeldahl analyses.

² Calculated from the equation of Brown, Batchelder and Scatchard (12) as modified by Scatchard (15):

$$\pi = \frac{26S(1 - 0.64g)c}{1 - (0.4 + 0.9pH)c}$$

where π = protein osmotic pressure, mm. Hg; c = protein concentration, g./cc; g = ratio of globulin to total protein. We assumed that in normal cat plasma $g = 0.42$, corresponding to an A:G ratio of 1.4. Electrophoretic studies of Deutsch and Goodloe (16) indicate that the actual A:G ratio in cat plasma is 0.7. However, a large proportion of the globulin fractions are proteins of low molecular weight which produce the osmotic equivalent of a higher A:G ratio. Kjeldahl analyses of the above samples after precipitation with Na_2SO_4 yielded 'apparent A:G ratios' of 1.3-2.0.

volume beyond the arterial system, for it is abolished if the arterioles are constricted with adrenaline. Nor is it present if the capillary pressure is maintained constant by simultaneous lowering of the venous pressure as shown in figure 4. The slow steady gain in weight (+0.26 grams/min.) is presumably a result of filtration of fluid from blood to tissues. When the arterial pressure was restored to its initial value, the vascular volume was also restored, following which the leg weight remained constant at a new level corresponding to the amount of fluid which had been filtered. The arterial pressure was then lowered below the value necessary to maintain constant weight. Exactly analogous changes of weight resulted except that they were of opposite sign. The slow steady loss of weight

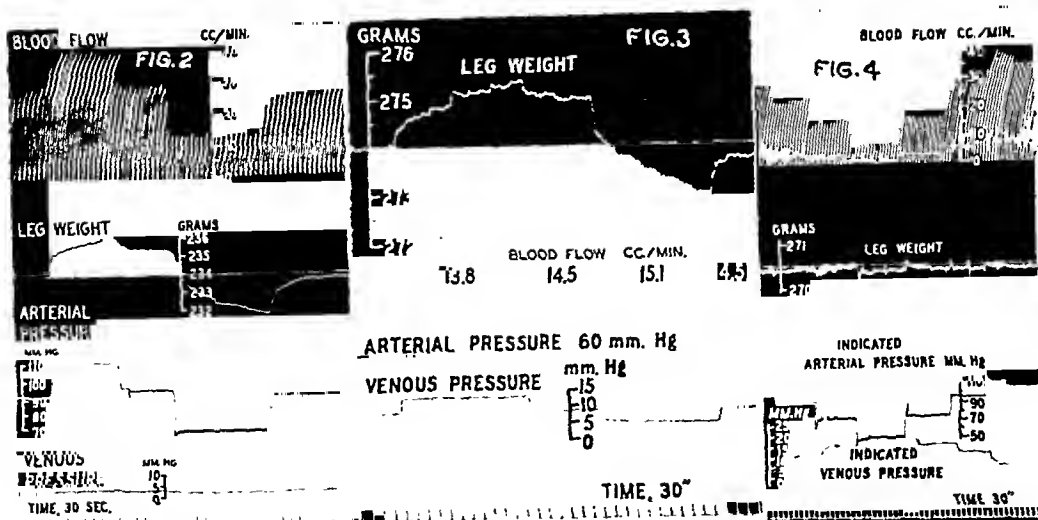


FIG. 2. THE EFFECTS OF ARTERIAL PRESSURE ON LIMB WEIGHT. A rise of arterial pressure above the isogravimetric value (91 mm. Hg) produced a sudden increase in limb weight (increased vascular volume) followed by a slow rate of gain of weight of $+0.26$ gram per minute (filtration rate). Exactly analogous changes, but of opposite sign, followed a fall in arterial pressure below the isogravimetric value (absorption rate -0.28 gram per minute). The time required to complete changes of vascular volume is variable and must be considered in determining the final slope. Slight alterations in venous pressure occurred owing to the changed pressure drop across the venous cannula at each different rate of blood flow; corrections for this variation are made as described in text. The arterial pressure scale is corrected for the pressure drop across the arterial cannula.

FIG. 3. THE EFFECTS OF VENOUS PRESSURE ON LIMB WEIGHT. A rise of venous pressure produced an initial small increment in limb weight (vascular volume) followed by a slow sustained rate of gain of weight of $+0.17$ gram per minute (filtration rate). Exactly analogous changes, but of opposite sign, followed a fall in venous pressure (absorption rate -0.21 gram per minute). The arterial and protein osmotic pressures remained constant. The time required to complete changes of vascular volume is variable and must be considered in determining the final slope. Note that the alterations in venous pressure, although small, produced detectable and reversible changes of blood flow as well as easily measurable rates of fluid transfer.

FIG. 4. THE ISOGRVIMETRIC STATE. This record illustrates the method of obtaining isogravimetric values for arterial pressure and blood flow. The tendency for each stepwise decrement in arterial pressure to cause absorption (as in fig. 2) is compensated for by increasing the venous pressure until the limb weight remains constant. In order to maintain the weight as constant as illustrated it was necessary to adjust the venous pressure within 0.3 mm. Hg and the arterial pressure within 2 mm. Hg. The isogravimetric capillary pressure and postcapillary resistance to blood flow are determined from the isogravimetric blood flow and the isogravimetric venous pressure as illustrated in figure 5. Indicated pressures must be corrected for the pressure drop across the cannulae at each rate of blood flow. Note that there are no changes of vascular volume such as those found with an uncompensated change of either arterial or venous pressures (figs. 2 and 3). This suggests that the principal site of change in vascular volume is the capillary bed; alternatively, changes in volume on the arterial side of the capillary circulation must be exactly counterbalanced by the changes in volume on the venous side of the capillary circulation.

(-0.28 grams/min.) is presumably a measure of the rate of absorption of fluid from tissues to blood.

DISCUSSION

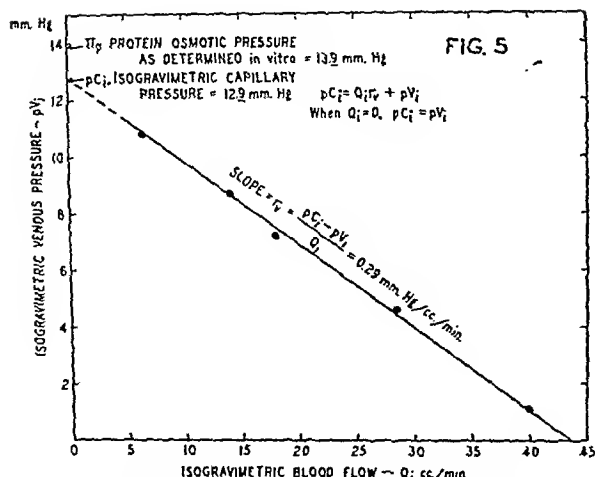
In the intact animal changes of arterial pressure are generally associated with complex changes in mean capillary pressure owing to simultaneous alterations in vascular tone. It is therefore not surprising that in the whole animal a rise of arterial pressure may cause filtration (17), absorption (18) or no detectable change in fluid balance (19). The results illustrated in figure 2 and in the remaining sections of this paper clearly demonstrate the quantitative relations between arterial pressure and rate of filtration or absorption when all other known factors are maintained constant.

2. *The effects of venous pressure on the limb weight.* Figure 3 shows the effects of variations in venous pressure on the limb weight, the arterial and protein pressures being maintained constant. The effects are similar to those produced by arterial pressure except that the changes of venous pressure required to produce comparable rates of filtration or absorption are one-fifth to one-tenth as great (compare fig. 3 with fig. 2). Thus an easily measurable rate of filtration is brought about by a rise of 4 mm. Hg in the venous pressure and a detectable effect results from a change of 0.5 mm. Hg or less.

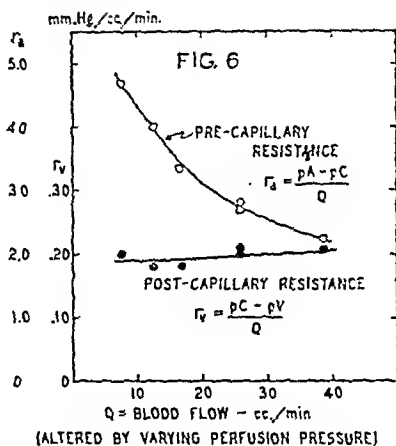
3. *The 'isogravimetric state.'* It will be clear from paragraphs 1 and 2 above that there exist an infinite number of pairs of values of arterial and venous pressures at which the leg will remain at constant weight. Thus the tendency to absorb, caused by a given reduction in arterial pressure, can be counterbalanced by raising the venous pressure until no net transfer of fluid occurs between blood and tissue (constant weight). Such an experiment, in which seven pairs of 'isogravimetric' arterial and venous pressures were obtained, is illustrated in figure 4. It is evident that if the arterial pressure is progressively lowered and the venous pressure raised to maintain constant weight there will come a point at which the venous pressure will equal the arterial pressure. Under these conditions there will be no pressure drop along the vascular tree and both the arterial and venous pressures will equal the capillary pressure. Since, by the terms of the experiment, there is no net transfer of fluid, this value of capillary pressure (isogravimetric capillary pressure) is equal and opposite to the sum of all pressures opposing filtration.

As pointed out by Green (20) the mean capillary pressure will be slightly less in the upper (ventral) half of the preparation than in the lower half. There will therefore be some transfer of fluid in the isogravimetric state even when the arterial and venous pressures are nominally equal. The average thickness of the hindlimb is about 2.5 cm. The mean capillary pressure in the top half is therefore about 0.5 mm. Hg less than in a horizontal plane bisecting the leg. This difference is by no means trivial since a change of 0.5 mm. Hg in mean capillary pressure regularly produces a detectable change in rate of fluid exchange in this preparation (section 2, fig. 9, table 3). In the isogravimetric state we therefore suppose that absorption occurs in the upper half of the preparation at a net rate of about 6 mgm. per minute per 100 grams tissue, while an equal rate of filtration takes place in the lower half even when arterial and venous pressures are nominally equal.

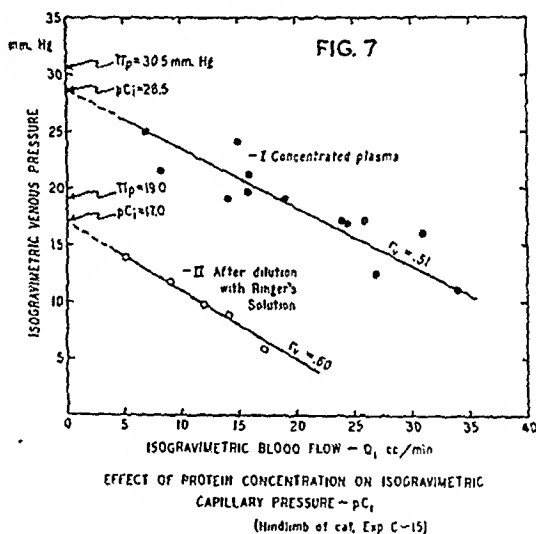
The isogravimetric capillary pressure cannot be obtained directly by equalizing arterial and venous pressures without disturbing the physiological properties of the capillaries because, when the arterial and venous pressures are equal, there is



DETERMINATION OF ISOGRVIMETRIC CAPILLARY PRESSURE AND POST-CAPILLARY RESISTANCE TO BLOOD FLOW Isolated hindlimb of cat, Exp. 23.



ANOMALOUS FLOW IN PRE-CAPILLARY CIRCULATION Hindlimb of cat, Exp. 25 Hematocrit = 32%



EFFECT OF PROTEIN CONCENTRATION ON ISOGRVIMETRIC CAPILLARY PRESSURE - p_{c_i} (Hindlimb of cat, Exp C-15)

FIG. 5. THE DETERMINATION OF ISOGRVIMETRIC QUANTITIES. Each point on the graph was obtained as illustrated in figure 4—the blood flow was altered by varying the arterial pressure and the venous pressure adjusted to maintain constant weight. At zero flow the pressure is everywhere equal along the vascular tree and, by the terms of the experiment, this pressure is equal and opposite to the sum of all pressures opposing filtration. At all finite values of blood flow the pressure at the arterial end of the capillary bed is greater than at the venous end; however, no net transfer of fluid occurs and the mean capillary pressure is presumably the same at all blood flows. The resistance to blood flow from the effective midpoint of the capillaries to the vein is, by definition, equal to the slope of the observed line; in contrast to precapillary resistance it is independent of rate of flow.

FIG. 6. THE RESISTANCE TO BLOOD FLOW from the femoral artery to the effective midpoint of the capillary circulation (precapillary resistance) increases as the flow is reduced by lowering the arterial pressure. This phenomenon may be a result of anomalous viscous flow of blood in the arterioles; the effect is diminished when the arterioles are dilated (21) or when most of the corpuscles are removed (7, 21) as described in text. The resistance to flow on the venous side of the capillary circulation (postcapillary resistance) is substantially independent of the flow rate.

no blood flow. However, its value may be estimated by plotting the difference between isogravimetric arterial and venous pressures (abscissa) against the isogravimetric arterial or venous pressures (ordinates) and extrapolating to zero pressure difference. The intercept on the ordinate is then the isogravimetric capillary pressure.

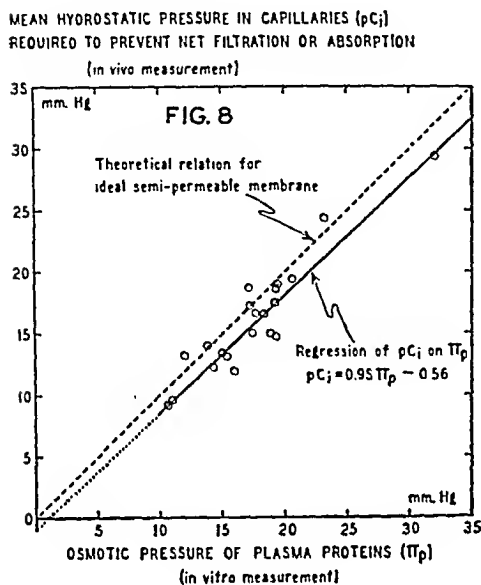
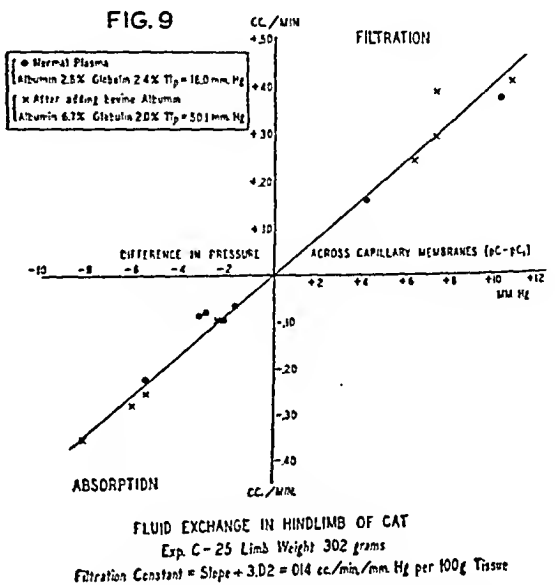


FIG. 8. THE EFFECTIVE OSMOTIC PRESSURE OF THE PLASMA PROTEINS in the hindlimb capillaries of cats and dogs. The mean hydrostatic pressure in the capillaries required to prevent net transfer of fluid is slightly less than the (in vitro) osmotic pressure of the plasma proteins over the entire range of protein concentrations so far investigated. The mean difference between these two quantities is 1.7 mm. Hg as compared with a standard error of measurement of 0.54 mm. Hg. In all probability this difference arises from a small concentration ($0.7 \pm .2\%$) of protein in tissue fluid bathing the external surfaces of the capillaries as explained in the text. At normal plasma protein pressures (23–27 mm. Hg) the effective osmotic pressure acting across the capillary membranes is 93 ± 1.5 per cent of the protein pressure as measured across a collodion membrane in vitro.

FIG. 9. FLUID EXCHANGE IN THE HINDLIMB OF A CAT. The rate of fluid exchange is simply proportional to the difference between the mean hydrostatic pressure in the capillaries ($p_c = Qr_v + p_v$) and the sum of all pressures opposing filtration (isogravimetric capillary pressure, p_{c_i}); it is independent of the absolute value of these quantities. The results of figures 8 and 9, taken together, show that the Starling Hypothesis may be applied with some precision to the hindlimbs of cats and dogs perfused under the specialized conditions of these experiments.

In practice this method of determining the isogravimetric capillary pressure is not the most precise one owing to the fact that the extrapolation is alinear. This alinearity will be discussed in greater detail below; it arises from the complex relations between pressure and flow which obtain when whole blood is employed for perfusion. The difficulty may be avoided by considering the relations between isogravimetric blood flow and isogravimetric venous pressure in the following analysis.



4. *The determination of capillary quantities.* Definition of symbols: the following notation will be employed in the remaining sections of this paper.

pA = arterial pressure, mm. Hg		of capillaries (pre-capillary resistance).
pV = venous pressure, mm. Hg		
pC = mean capillary pressure, mm. Hg	$r_v = (pC - pV) \div Q$	= resistance to blood flow from effective midpoint of capillaries to vein (post-capillary resistance).
π_p = osmotic pressure of plasma proteins, mm. Hg		
π_t = osmotic pressure of proteins in tissue fluid, mm. Hg	i	= subscript referring to isogravimetric conditions.
Q = blood flow, cc./min.		
$r_a = (pA - pC) \div Q$	$\pm F$	= rate of filtration (+) or absorption (-), grams/min.
= resistance to blood flow from artery to effective midpoint		

Rearranging the definition of r_v we have, in the isogravimetric state

$$pC_i = Q r_v + pV_i$$

In order to solve for pC_i , plot Q_i (abscissa) against pV_i (ordinate) from data similar to that shown in figure 4. Extrapolate to zero flow; when $Q_i = 0$, $pC_i = pV_i$. Such a plot is shown in figure 5. It is seen that the relation between Q_i and pV_i is linear over a wide range of values and that the extrapolation to zero flow may be made with an uncertainty of less than 1 mm. Hg. The slope of the line is r_v , which is constant and independent of flow.

Presumably both filtration and absorption occur at all finite values of blood flow in the isogravimetric state. Thus when pV_i is small and Q_i large (fig. 5), the pressure drop along the capillaries is also large; filtration occurring at the arterial ends or along the entire length of some capillaries is counterbalanced by equal absorption at the venous ends or along the entire length of other capillaries. But as the flow is reduced the pressure drop in the capillaries is also reduced until, at zero flow, the pressure is everywhere equal along the capillary and no fluid exchange takes place along its length. It is of importance for the subsequent development in this paper to note that the value of r_v is unaltered by the changes of flow or of filtration and absorption.

A similar analysis can be made on the arterial side of the capillary circulation using the definition $pC_i = pA_i - Q r_a$. In this case, however, it is found experimentally that the relation between Q_i and pA_i is not linear. Since pC_i is constant (isogravimetric conditions) it follows that r_a , the precapillary resistance to blood flow, must vary with flow. This is illustrated in the experiment of figure 6 in which the precapillary resistance was more than doubled as the flow was decreased by reducing the perfusion pressure. The postcapillary resistance (r_v), determined simultaneously, remained constant.

DISCUSSION

This increase in precapillary resistance at low flows is not unexpected. It was found by Pappenheimer and Maes (21) and by Green *et al.* (22) that the overall resistance to blood flow ($r_a + r_v$) increases greatly at low flows and that the effect is most prominent in constricted vessels. The former workers attributed the

effect to the anomalous viscous properties of the blood, for it was not present when Ringer's solution was substituted for blood. The data now presented (fig. 6) show that the entire effect is localized in blood vessels proximal to the effective midpoint of the capillaries. It may be inferred that the blood vessels on the venous side of the capillary circulation have dimensions such that anomalous flow effects are too small to be of significance; in this respect they resemble fully dilated arterioles. Six of the present series of experiments have been conducted with plasma containing relatively few corpuscles (hematocrit about ten per cent). Under these conditions the precapillary resistance (r_a) varied only slightly with changes of flow, thus confirming and extending the earlier observations with Ringer's solution.

While the resistance to blood flow is not the principal subject of this paper, it may be pointed out that the resistances to flow on either side of the capillary circulation have not previously been measured, although they are major factors determining the mean capillary pressure and the rate of fluid transfer as pointed out by Bayliss and Starling (23). The dependence of mean capillary pressure on arterial and venous tone may be stated quantitatively by combining the definitions of r_a and r_v given above and solving for pC .

$$pC = \frac{\frac{r_v}{r_a} pA + pV}{1 + \frac{r_v}{r_a}}$$

It is seen that at any given values of arterial and venous pressures the mean capillary pressure depends solely on the *ratio* of the postcapillary to precapillary resistances to blood flow. Since r_v and r_a are now measurable the way is open for the quantitative study of factors affecting the mean capillary pressure.

5. *Comparison of the isogravimetric capillary pressure with the osmotic pressure of the plasma proteins.* The isogravimetric capillary pressure determined as described above is equal to the sum of all pressures opposing filtration. It is of interest to compare its value with the osmotic pressure of the plasma proteins as determined with an artificial semipermeable membrane *in vitro*. Figure 7 shows the effects of alterations in protein concentration on isogravimetric quantities in one experiment. It is seen that the protein osmotic pressure as measured *in vitro* (π_p) was approximately 2 mm. higher than the isogravimetric capillary pressure at each of the two protein concentrations employed. Note that the postcapillary resistance to blood flow (r_v) remained approximately constant throughout the measurements; alterations in postcapillary resistance, produced by the infusion of drugs or other procedures, do not affect the value of pC ; if the protein concentration is constant.

The results of 22 such comparisons of pC with π_p are summarized in figure 8. The upper (broken) line represents the theoretical values which would obtain if the isogravimetric capillary pressure were equal and opposite to the (*in vitro*) protein pressure. The lower solid line is the best straight line drawn through the experimental points (method of least squares). It is seen that the capillary hydrostatic pressure in the isogravimetric state is, on the average, one to 2 mm.

Hg less than the protein pressure as measured in vitro over the range of protein pressures so far investigated. Thus,

$$pC_i = 0.95\pi_p - 0.56 \quad S.D. = \pm .6 \text{ mm. Hg}$$

$$S.E. = S.D. \div \sqrt{n} = 0.34 \text{ mm. Hg.}$$

If no other forces (e.g., tissue pressure) were involved, the results of figure 8 could be explained on the basis of a concentration of protein in tissue fluid sufficient to exert an osmotic pressure of 1 mm. Hg in the lower range of protein concentrations and 2 mm. Hg in the higher range. It may be noted that tissue pressure would act to increase pC_i with respect to π_p . Thus if the protein concentration were reduced to zero we should expect that a *positive* hydrostatic pressure in the capillaries would be required to counterbalance tissue pressure. Actually the extrapolation to zero protein pressure (fig. 8) intercepts the capillary pressure axis at a negative value (-0.6 mm. Hg), a result which would be difficult to explain in terms of tissue pressure but which would be expected if a small concentration of protein remained in the tissue fluid. Further evidence will be given below (Section 6B) that the tissue pressure is in fact negligible under the conditions of these experiments and we make the provisional conclusion that the isogravimetric capillary pressure is equal and opposite to the effective osmotic pressure of the plasma proteins.

$$pC_i = (\pi_p - \pi_t) = \text{effective protein pressure across capillary membranes.}$$

If this conclusion is correct we have for normal plasma ($\pi_p = 25$ mm. Hg) $\frac{pC_i}{\pi_p} = \left(0.95 - \frac{.56}{25}\right) = 0.93$, whence the effective osmotic pressure of the plasma proteins within the living capillaries is 93 per cent (standard error ± 1.5 per cent) of the osmotic pressure measured with a collodion membrane in vitro. The mean value of π_t under these conditions is 1.7 ± 0.4 mm. Hg, a pressure which would be expected in fluid containing $0.8 \pm .2$ grams plasma protein per 100 cc. This estimate of protein in tissue fluid is too high if albumin and low molecular weight globulins constitute the principal protein fractions of the capillary filtrate. Electrophoretic studies of serum exudates (24) and of lymph (25) suggest that a partial sieving action does occur, and in this case our estimate of protein in fluid bathing the capillary walls would be closer to 0.7 ± 0.2 per cent.

The close correspondence between isogravimetric capillary pressure and plasma protein pressure is maintained only in the first few hours of perfusion and the data given above were obtained in this period. After 4 to 6 hours the isogravimetric capillary pressure diminishes; in a typical experiment it remained within 90 to 95 per cent of the plasma protein pressure for the first four hours of perfusion, but fell to 70 per cent in the fifth hour and to 30 per cent after six hours of perfusion. Presumably this is a quantitative expression of failure of the capillary membranes to retain plasma protein after prolonged perfusion.

DISCUSSION

The isogravimetric capillary pressure is, on the average, 1.7 ± 0.4 mm. Hg less than the osmotic pressure of the plasma proteins and this value is equivalent to

a protein concentration of $0.7 \pm .2$ per cent in fluid bathing the external surfaces of the capillaries. Inspection of table 2 shows that this concentration is slightly greater than estimates of protein in capillary filtrate (26) or in lymph obtained during venous congestion (27). On the other hand it is considerably less than the protein concentration in small samples of lymph collected during massage of the resting dog's leg (28). It is clear that these results do not confirm the view, originally expressed by Starling (1) and repeatedly stated by Drinker (29-31), that the composition of tissue fluid is identical with that of lymph. Indeed, it appears unlikely that the protein concentration of tissue fluid is at any time uniform throughout the interstitial spaces. The diffusion coefficients of the plasma proteins are such that relatively large concentration gradients are possible be-

TABLE 2. PROTEIN COMPOSITION OF TISSUE FLUIDS

FLUID	PROTEIN	NUMBER OF OBSERVATIONS	REFERENCES
1. Capillary filtrate in human forearm during venous congestion (60 mm. Hg)	$c = 0.3 \pm 0.3\%$	4	(26)
2. Lymph from dog's leg during venous congestion (40 to 60 mm. Hg)	$c = 0.2\%$	1	(27)
3. Average tissue fluid bathing external capillary surface in perfused limbs of cats and dogs	$\pi = 1.4 \pm 0.4$ mm. Hg. $c = 0.7 \pm 0.2\%$	22	Present data
4. Lymph obtained during massage of quiescent limb (dog)	$\pi = 17.5 \pm .8$ mm. Hg. $c = 1.9 \pm 0.2\%$	8	(28)

¹ This value is larger than would be predicted from the observed protein concentration. The osmotic pressure of a 1.9 per cent solution of pure albumin in .15 molar NaCl at pH 7.3 is 6.1 mm. Hg (13) and that of a 1.9 per cent solution of plasma proteins is less than 5.0 mm. Hg.

tween tissue fluid undergoing absorption at the venous end of the capillaries and tissue fluid composed of freshly formed capillary filtrate at the arterial end of the capillary. Even if all filtration and absorption processes were stopped, some 20 minutes would be required to reach 90 per cent equalization of protein concentration over a distance of 50 microns. As pointed out by Landis *et al.* (26), the protein content of lymph may lie anywhere between that of capillary filtrate (as in venous congestion) and that of tissue fluid immediately adjacent to capillary areas taking part in the absorptive process. On this hypothesis our estimate of protein in tissue fluid represents an average concentration lying between these two extremes.

6. *Filtration and absorption.* A. THE FILTRATION COEFFICIENT. Once having determined r_s in the isogravimetric state, it is possible to compute the value of the mean hydrostatic pressure in the capillaries when the arterial or venous pressures are altered so as to produce *net* filtration or absorption as in figures 2 and 3. Thus the value of r_s was found to be independent of the blood flow (figs.

5, 6 and 7) and of changes in filtration or absorption of fluid along the capillary wall (section 4). The mean hydrostatic pressure in the capillaries following an uncompensated change of arterial or venous pressure is then given by the relation

$$pC \approx Qr_o + pV$$

where r_o is obtained in the isogravimetric state but Q and pV are no longer isogravimetric quantities.

The mean pressure head across the capillary membranes available for filtration or absorption is $pC - pC_i$; for the latter term is equal to the sum of all pressures opposing filtration. Figure 9 shows the relation between the pressure head across the membranes and the rates of net filtration ($+F$) or net absorption ($-F$). The rate of fluid exchange is simply proportional to the pressure difference across the capillary membranes. Black circles are points obtained with undiluted blood with a measured osmotic pressure of 16.0 mm. Hg. The crossed points were obtained after concentrated bovine albumin was added until the protein osmotic pressure was 50.1 mm. Hg. The two sets of points do not differ significantly.

TABLE 3. FILTRATION COEFFICIENTS OF PERFUSED HINDLIMBS

SPECIES	FILTRATION COEFFICIENT K = GRAMS/MIN/MM Hg/100 GRAMS TISSUE		
	Mean	Error of mean	Standard deviation
Cats (14).....	0.012	0.0007	0.0027
Dogs (4).....	0.014	0.0006	0.001

The rate of fluid exchange is therefore independent of the absolute values of capillary and protein pressures and depends only on the *difference* between the mean capillary pressure (pC) and the effective protein osmotic pressure (pC_i). Thus,

$$\pm F \approx K(pC - pC_i)$$

where the proportionality factor (K) may be termed the 'filtration coefficient' of the capillary membranes. The mean value of K in the hindlimbs of 14 cats was 0.012 grams per minute per mm. Hg pressure difference across the capillary membranes per 100 grams tissue. The value was not significantly different in the hindlimbs of four dogs (table 3).

DISCUSSION

The results of figure 9 show that the rate of flow of fluid passing in either direction across the capillary membranes is simply proportional to the difference between the mean hydrostatic pressure in the capillaries and the sum of all pressures opposing filtration. Evidence has been given above that the latter term is equal to the effective osmotic pressure of the plasma proteins. These results, taken together, show that the Starling Hypothesis may be applied with some precision to the hindlimbs of cats and dogs perfused under the specialized conditions of these experiments.

The values of table 3 may be compared with estimates of filtration rate in the intact human forearm. Landis and Gibbon (32) found that a rise in venous pressure of 10 cm. H_2O produced, on the average, a filtration of 0.033 cc. per 100 cc. forearm per minute. Assuming that a rise in venous pressure of 10 cm. H_2O produces an increment of 8 cm. H_2O in mean capillary pressure, the 'filtration coefficient' of the human forearm would be 0.0055 gram/min/mm. Hg/100 grams tissue or less than half that of the isolated perfused hindlimb of the cat or dog. This difference may be an expression of a smaller capillary surface per unit volume of tissue in the human forearm, a difference which is suggested also by the absolute value of the blood flow, which is generally reported to be less per unit tissue volume in the human forearm (33-35) than in the extremities of dogs or cats (36-38).

Proportionality between pressure head and flow is characteristic of the viscous flow of fluids through artificial porous membranes. From this point of view it will be interesting to determine if the filtration coefficient of the capillary membranes varies inversely with the viscosity at different temperatures as found with artificial porous membranes (39).

B. THE EFFECTS OF PROLONGED FILTRATION AND ABSORPTION. The results illustrated in figures 1 and 2 show that net filtration or absorption of small quantities of fluid (less than one gram per 100 grams tissue) occur at a constant rate following an increment or a decrement in mean capillary pressure. Evidence has been given by Krogh, Landis and Turner (40) and by Landis and Gibbon (32) that continued net filtration increases tissue pressure in the human forearm, thereby causing a progressive decrease in the rate of filtration following an increment in venous pressure. Continued net absorption, on the other hand, must eventually be limited by the volume and protein content of the fluid originally present in the tissue spaces, a limitation which was clearly envisioned by Starling (1) but which has never been evaluated experimentally.

Figure 10 shows the relations between the volume of tissue fluid and the total pressure opposing filtration (isogravimetric capillary pressure) in an isolated hindlimb of a dog. The design of the experiment was as follows. The tissue fluid was first concentrated by allowing net absorption to take place; capillary pressure was then increased above the isogravimetric value and 68 grams of fluid filtered from plasma to tissue fluid. The filtration process was interrupted at intervals in order to obtain isogravimetric values. Approximately five hours were required to complete the experiment.

It is seen that the isogravimetric capillary pressure obtained at minimum limb weight was only two-thirds of the plasma protein pressure. Presumably this is a result of the previous prolonged absorption with resulting concentration of protein in tissue fluid and diminution of effective osmotic pressure of plasma proteins. Filtration of 15 grams of fluid into the tissue spaces diluted the tissue fluid proteins by an amount sufficient to increase the isogravimetric pressure to 92 ± 8 per cent of the plasma protein pressure. Further filtration of 33 grams did not increase this value significantly and in this latter range of tissue fluid volumes the rate of filtration remained constant following an increment in capillary pressure.

In another experiment of this type filtration was continued until 175 grams of fluid had been filtered into the tissue spaces. (65 per cent of the initial weight of the limb). Conditions were made such that the osmotic pressure of the proteins was only a small fraction of the total filtration pressure by diluting the plasma with a large volume of Ringer's solution and raising the mean capillary pressure to an abnormally high value. The results are summarized in table 4.

It is seen that the filtration rate remained constant until 90 grams of fluid had been filtered. The limb appeared moderately edematous at this time.

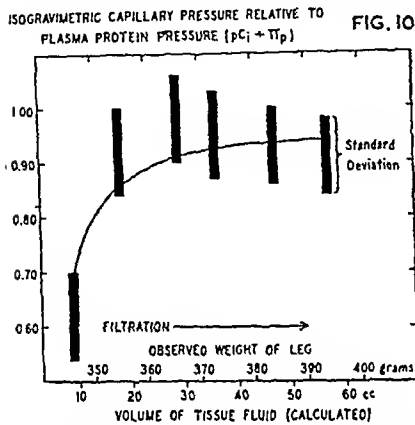


FIG. 10

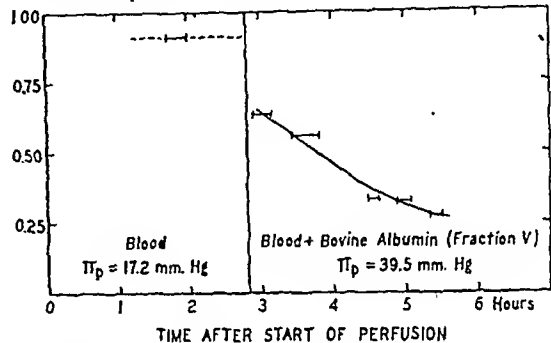
 EFFECTIVE OSMOTIC PRESSURE
RELATIVE TO PLASMA PROTEIN PRESSURE
 $pC_i \div \pi_p$


FIG. 11

FIG. 10. THE EFFECTS OF PROLONGED FILTRATION IN PREVIOUSLY DEHYDRATED LIMB. The isogravimetric capillary pressure rises rapidly at small tissue fluid volumes but remains relatively constant over a wide range of higher tissue fluid volumes. Presumably this is the result of dilution of protein in tissue fluid with capillary filtrate, which must contain protein in concentration less than that of tissue fluid at the end of the filtration period (0.5%). The smooth curve and the tissue fluid volume scale are calculated from the relation $V = (G_o + [P]_f \cdot \Delta W) \div [P]_t$ as explained in the text.

FIG. 11. THE EFFECTIVE PROTEIN OSMOTIC PRESSURE RELATIVE TO TRUE PROTEIN OSMOTIC PRESSURE following the addition of bovine albumin to the perfusing blood. Bovine albumin, unlike concentrated homologous plasma, is not retained by the capillary membranes in the hindlimbs of cats or dogs perfused under the conditions of these experiments.

Further filtration occurred at a progressively slower rate and this we attribute to developing tissue pressure for the protein pressure opposing filtration remained a small fraction of the mean capillary pressure. Evidently the isolated hindlimb differs from the human forearm in that tissue pressure does not become a significant factor opposing filtration until the limb is grossly edematous.

The results illustrated in figure 10 may be employed to estimate the volume of tissue fluid and the protein composition of the filtrate. Thus,

$$V = \frac{G_o + [P]_f \Delta W}{[P]_t}$$

where V = volume of tissue fluid, G_o = total quantity of protein in tissue fluid at start of filtration, $[P]_f$ = concentration of protein in capillary filtrate, $[P]_t$ = concentration of protein in tissue fluid = $\psi(\pi_p - pC_i)$, ψ = relation between

protein concentration and protein osmotic pressure given in table 1 and ΔW = increase in weight of limb during filtration.

This equation was employed to construct the 'tissue fluid volume' scale on the abscissa of figure 10. The protein concentration in capillary filtrate corresponding to the calculated curve is 0.3 per cent; this value best fits the observed data but other values, within the range 0.2–0.4 per cent, lie within the range of analytical errors.

DISCUSSION

Starling (1) considered the protein in tissue fluid to be an important factor in the regulation of the fluid exchange. To quote (p. 394): "With diminished capillary pressure there will be an osmotic absorption of salt solution from the extravascular fluid, until this becomes richer in proteids; and the difference between its (proteid) osmotic pressure and that of the intravascular plasma is equal

TABLE 4. PROLONGED FILTRATION AND EDEMA FORMATION
Mean capillary pressure 53 ± 3 mm. Hg. Protein pressure
9–13 mm. Hg. Initial weight of limb 270 grams

TIME	AMOUNT FILTERED	FILTRATION RATE GRAMS/ MIN. IN 20-MINUTE PERIOD	VISIBLE EDEMA
<i>minutes</i>	<i>grams</i>		
0	0	—	None
20	45	2.25	+
40	90	2.25	++
60	126	1.80	+++
80	155	1.45	++++
100	171	0.80	++++

to the diminished capillary pressure." A consideration of figure 10 shows that concentration of extravascular protein does in fact occur, but only after relatively large quantities of tissue fluid have been absorbed. In the intact animal the effective osmotic pressure would be diminished by dilution of the plasma proteins long before the concentration of protein in tissue fluid became a significant factor. It therefore appears likely that the principal osmotic factor regulating the fluid exchange normally involves changes in plasma protein concentration rather than changes in the protein concentration of extravascular fluid.

7. *Foreign protein.* In four experiments (three cats, one dog) bovine albumin was added to the perfusion reservoir. Two preparations of albumin were employed, a) Armour Co. 'Fraction V' which is said to contain about three per cent of impurities in the form of globulin fractions and acetate buffer; b) crystalline bovine albumin prepared by the Armour Co. The albumin was added in a 25 per cent solution titrated to pH 7.4. The results were similar for both preparations.

Figure 11 shows the effective osmotic pressure relative to plasma protein pressure before and after the administration of albumin. One hour after the addition

of albumin the effective osmotic pressure across the capillary membrane was only 50 per cent of the protein pressure, a value which would obtain if the protein in tissue fluid were sufficient to exert an osmotic pressure of 20 mm. Hg (equivalent to 4.9 per cent albumin). Evidently purified or crystalline bovine albumin, unlike concentrated homologous plasma, is not retained by the capillary membranes in the hindlimbs of cats and dogs perfused under the conditions of these experiments. The reasons for this leakage are not clear; Heyl, Gibson and Jane-way (41) found that crystalline bovine albumin is as effective as reconstituted human plasma in the restoration of blood volume following hemorrhage in humans.

Despite the large and progressive leakage of bovine albumin through the capillary membranes, the *filtration coefficient* remained unaltered. This is illustrated in figure 9 in which the net rate of fluid exchange for any given difference between mean capillary pressure and effective osmotic pressure was the same before and after albumin was added, although the effective osmotic pressure after the addition of albumin diminished progressively from 40 to 15 mm. Hg. The filtration coefficient was similarly unaffected by the leakage of protein which occurred at the end of each experiment as described in section 5 above. Evidently the permeability of the capillary wall to fluid is not necessarily related to the permeability to protein in the mammalian circulation.

SUMMARY

1. Methods are described for determining the following quantities in isolated perfused hindlimbs of cats and dogs.

- a) Mean capillary pressure (accuracy ± 0.5 mm. Hg),
- b) Total pressure opposing net filtration of fluid from plasma to tissues (isogravimetric capillary pressure),
- c) Net rate of fluid transfer across capillary membranes,
- d) Resistance to blood flow from femoral artery to effective midpoint of capillary circulation and from capillary circulation to the femoral vein.

2. The rate of net fluid exchange between plasma and tissue spaces may be delicately adjusted over a wide range of values by varying the arterial pressure (fig. 2), the venous pressure (fig. 3) or the protein osmotic pressure of the plasma. At any given protein pressure there are an infinite number of pairs of values of arterial and venous pressures at which no net transfer of fluid occurs; two or more pairs of such values define the mean hydrostatic pressure in the capillaries (fig. 5).

3. The mean hydrostatic pressure in the capillaries at which no net transfer of fluid takes place (pC_i) is 93 ± 1.5 per cent of the normal osmotic pressure of the plasma proteins (π_p). In the range of plasma protein pressures, 8 to 32 mm. Hg, the relation is given by

$$pC_i = 0.95\pi_p - 0.56 \quad (\text{fig. 8}).$$

Reasons are given for believing that pC_i is equal and opposite to the effective osmotic pressure of the plasma proteins.

4. The mean pressure head available for net fluid transfer across the capillary

membranes is the mean capillary pressure minus the isogravimetric capillary pressure ($pC - pC_0$). The rate of filtration or absorption is accurately proportional to this pressure head and is independent of the absolute protein or capillary pressures over a wide range of values (fig. 9). The proportionality constant is termed the 'filtration coefficient'; its mean value in the hindlimbs of 16 cats was 0.012 grams per minute per mm. Hg pressure difference across the capillary membrane per 100 grams tissue (table 3).

5. Absorption of fluid following a decrement in capillary pressure continues at a constant rate until the proteins in tissue fluid become sufficiently concentrated to oppose continued absorption. However, the quantity of protein present in tissue fluid is so small that a large volume of tissue fluid must be absorbed before the protein osmotic pressure of tissue fluid is significantly increased. It therefore appears likely that the principal osmotic factor regulating the fluid exchange normally involves changes in plasma protein concentration rather than changes in the composition of tissue fluid.

6. Filtration of fluid following an increment in capillary pressure continues at a constant rate until the limb becomes grossly edematous (table 4). In contrast to the intact human forearm, tissue pressure does not limit the rate of filtration over a wide range of tissue fluid volumes.

7. Crystalline bovine albumin, unlike concentrated homologous plasma, is not retained by the capillary membranes in the hindlimbs of cats or dogs perfused under the conditions of these experiments (fig. 11).

8. The resistance to blood flow from the femoral artery to the effective midpoint of the capillary circulation increases greatly when the blood flow is diminished by lowering the perfusion pressure (fig. 6). The effect is perhaps a result of anomalous viscous flow of blood in the arterioles; it is diminished or absent when the blood contains less than ten per cent red cells. The resistance to blood flow on the venous side of the capillary circulation is independent of flow even at normal corpuscular concentrations (figs. 5, 6 and 7). It is suggested that the dimensions of the venules are such as to minimize the effects of anomalous flow.

It is a pleasure to thank Prof. E. M. Landis, Prof. G. Scatchard, Dr. J. L. Oncley, Dr. S. W. Armstrong, Jr., Dr. D. M. Hegsted and Prof. C. A. Janeway for their help and suggestions in connection with these experiments. It is a pleasure also to acknowledge the technical assistance of Mr. Angelos Afentakis.

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FORCE AND TIME ELEMENTS IN CIRCULATORY CHANGES UNDER ACCELERATION: CAROTID ARTERIAL PRESSURE DEFICIENCY AREAS¹

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Received for publication February 14, 1948

Acceleration, as developed by means of a centrifuge, has become a very useful tool in biological research. Large displacements of movable components of the living organism may be brought about by such a force transmitted to the organs and tissues. Several aspects of the effects of acceleration on the circulatory system have already been reported (1, 2). In the present study an evaluation of the relative effects on the arterial pressure of g force as a function of time has been made.

EXPERIMENTAL

Apparatus and methods have been previously described (2). The experiments were carried out on a centrifuge of 10-foot radius. Dogs and monkeys (*M. rhesus*) under local or general (usually amytal) anesthesia were used. Blood pressure in the carotid and femoral arteries was recorded by means of an electric resistance manometer. E.C.G., E.E.G. and respiratory rate were also commonly recorded; these served to determine various bodily changes and as a guide to the animal's condition. Most of the experimental runs were 5, 10, 15, 20, 25 and 30 seconds in duration at both positive (chiefly) and negative acceleratory forces of 1, 2, 3, 4 and (sometimes) 6 g. About 550 tests were carried out on dogs and about 450 on monkeys.

In earlier work on the circulatory changes which occur under exposure to high accelerations we considered among other features arterial pressure and flow relationships. Further useful and perhaps more significant measure of the circulatory disturbance may be made on the basis of the overall rise or fall in arterial pressure from the base level in time during and immediately after acceleratory test. Such a shift may be determined by measurement on a graph of the area circumscribed by the experimental blood pressure curve connected by a line drawn horizontally from the initial (basal) to the final blood pressure level at or shortly after the end of centrifugation. The circulatory change thus found may be described as the pt_p area, in which p represents arterial pressure change (mm. Hg), and t_p the time (seconds) from the first rise or fall in blood pressure until its return to normal. Determination of this area was accurately made with a planimeter. The acceleratory area (gt_e) was measured similarly (fig. 1).

¹ Work carried out under a contract with the U. S. Navy, Office of Naval Research, and the University of Virginia.

An increase or decrease in arterial pressure under centrifugation may hardly be considered from the simple credit or debit viewpoint. It is nevertheless clear that reduction in blood pressure in arterial vessels supplying the head may constitute a serious functional loss or 'deficiency', and this term has been used in pertinent parts of this report. Emphasis has been made in a previous report of the significance of time and intensity factors in exposure to g forces (3).

RESULTS

Carotid arterial pressure deficiency. Average curves indicating the effect of various intensities and durations of g on the carotid arterial pressure of the monkey and dog are given in figures 2 and 3. The monkey showed a marked post-centrifugal increase in blood pressure above the normal level; it rose to a peak

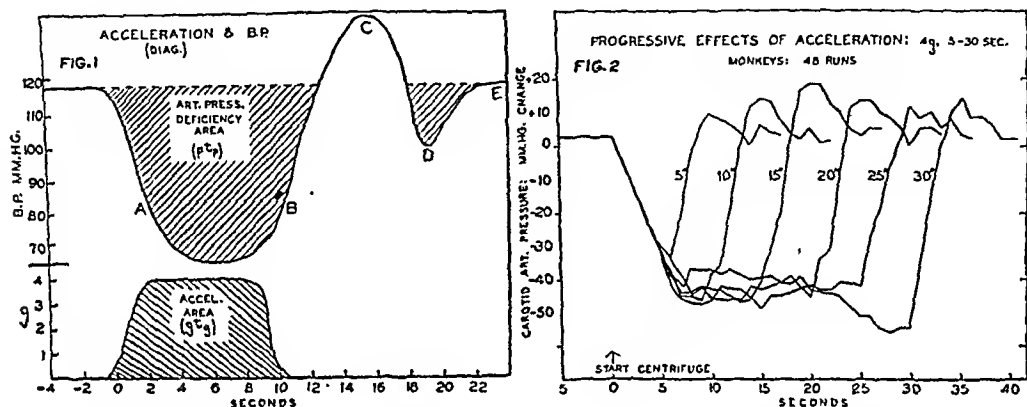


FIG. 1. GRAPH SHOWING COMPONENTS of carotid arterial pressure change during and after exposure to acceleratory forces (diagrammatic, monkey).

FIG. 2. CAROTID ARTERIAL PRESSURE CHANGES in the monkey during and after progressively increasing time exposures to 4 g. Arterial pressure levels, recorded continuously, are marked from second to second. Note post-acceleratory rises above the normal in blood pressure.

at about 15 seconds, and fell off thereafter. In the dog there was usually no similar rise (or only a slight one) in arterial pressure (fig. 3).

The data presented in table 1 demonstrate that for the dog the time required for the carotid arterial pressure to return to normal after centrifugation, at levels of 1, 2 and 4 g, was independent of the actual duration of the centrifuge test. However, $t_p - t_g$ was directly proportional to the magnitude of the acceleratory force to which the animal had been subjected. In the case of the monkey, the time interval $t_p - t_g$ was found to be practically independent of both duration and intensity of the exposure.

In figure 4 the pt_p areas for both animals tested were graphed against the corresponding gt_g areas. The results showed in each case a straight line relationship: that for the monkey was given by the equation $pt_p \text{ area} = 11.5 gt_g \text{ area}$, and for the dog by $pt_p \text{ area} = 21.5 gt_g \text{ area}$.

In the case of the dog a post-centrifugal fall in carotid arterial pressure was

not generally observed following runs of 1 g; but at 2 and 4 g, post-acceleratory blood pressure deficiency areas were noted and measured (fig. 3; table 2). This secondary fall in arterial pressure seemed to be independent of the duration of the run, but increased directly with the intensity of g.

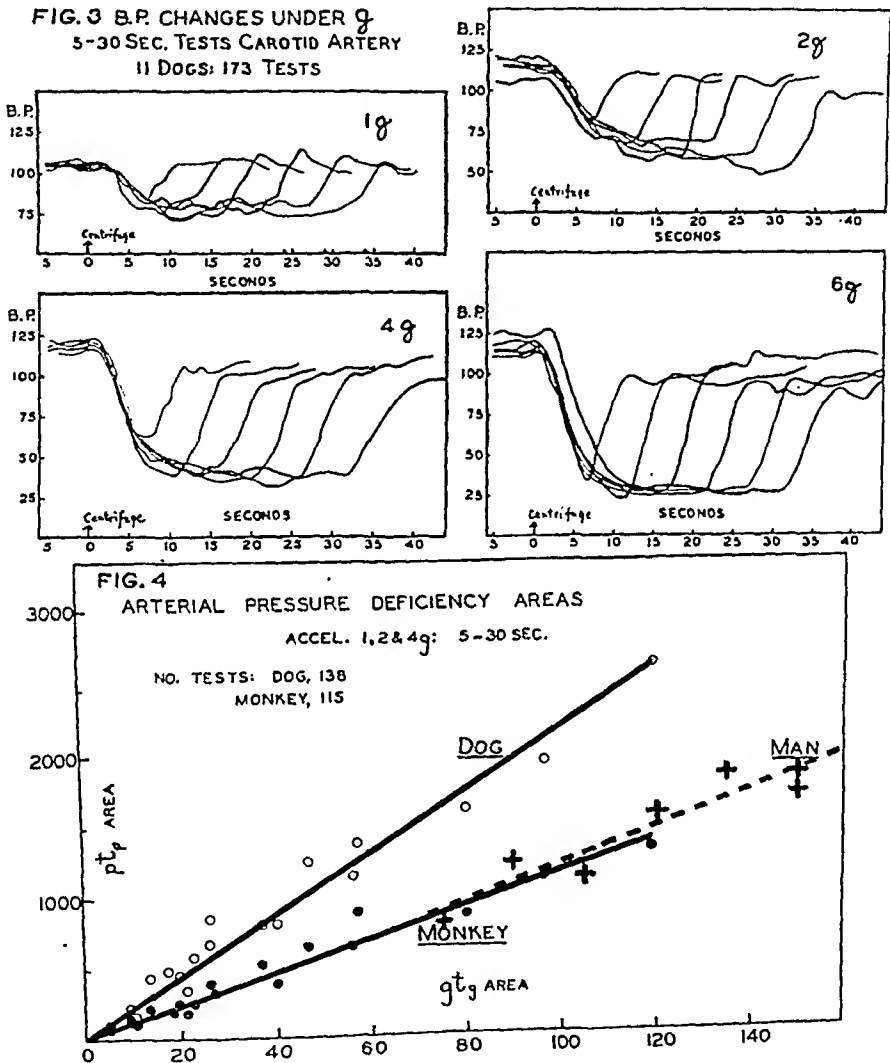


FIG. 3. CAROTID ARTERIAL PRESSURE CHANGES in the dog under different times and intensities of g. No post-acceleratory rises in blood pressure above normal occur in the dog, in contrast to the monkey (fig. 2).

FIG. 4. ARTERIAL PRESSURE DEFICIENCY AREAS compared in monkey and dog. Under given conditions, far greater circulatory shifts occur in the dog than in the monkey (see text).

The above observations led to the investigation of the $pt_p:gt_g$ relationship from a somewhat different point of view. Four dogs were exposed in 43 experiments to different g forces for varying periods of time, so that the product of g and t_g in all cases equalled 48 g-sec. Carotid arterial pressure curves were secured

TABLE 1. CAROTID ARTERIAL PRESSURE DEFICIENCY AREAS UNDER ACCELERATION
Animals subjected to various intensities and durations of g

No. of tests: monkey, 115; dog, 138

ACCELERATION	ACTUAL TIME OF EXPOSURE	TIME FROM INITIAL DROP OF B.P. TO ITS RETURN TO NORMAL		ACCELERATORY AREAS	$t_p - t_g$		ARTERIAL PRESSURE DEFICIENCY AREAS	
		Dog	Monkey		Dog	Monkey	Dog	Monkey
g	t_g sec.	sec.	t_g sec.	$g t_g$			$p t_p$	$p t_p$
1	7.4	9.0	8.7	5.0	1.6	1.3	101	76
1	13.1	13.5	14.0	9.6	0.4	0.9	223	173
1	17.3	18.5	17.6	13.5	1.2	0.3	423	290
1	22.1	23.5	23.0	18.2	1.4	0.9	478	233
1	28.0	28.0	27.0	22.5	0.0	-1.0	573	304
1	32.5	33.0	33.3	26.9	0.5	0.8	844	337
2	7.8	12.5	6.9	10.2	4.7	-0.9	171	131
2	13.1	21.5	12.2	19.5	8.4	-0.9	502	233
2	18.0	21.5	18.0	26.0	3.5	0.0	688	348
2	23.0	31.0	23.5	37.0	8.0	0.5	878	583
2	28.0	34.0	26.7	46.5	6.0	-1.3	1,290	633
2	33.1	37.5	35.0	57.0	4.4	1.9	1,420	839
4	7.4	19.0	9.4	21.0	11.6	2.0	456	234
4	12.7	25.0	14.8	40.0	12.3	2.1	938	475
4	17.2	25.5	18.0	56.0	8.3	0.8	1,270	785
4	23.0	34.5	25.2	80.0	11.5	2.2	1,760	1,020
4	28.0	40.5	32.8	96.7	12.5	4.8	2,070	1,350
4	34.0	51.0	36.1	119.0	17.0	2.1	2,790	1,530

TABLE 2. POST-ACCELERATORY CAROTID ARTERIAL PRESSURE DEFICIENCY AREAS

Dog: 138 runs

g	TIME OF EXPOSURE	POST-CENTRIFUGAL A.P. DEFICIENCY AREAS	g	TIME OF EXPOSURE	POST-CENTRIFUGAL A.P. DEFICIENCY AREAS
	sec.	$p t_p$		sec.	$p t_p$
1	5-30	0	4	5	108
2	5	(3)	4	10	134
2	10	39	4	15	128
2	15	16	4	20	188
2	20	73	4	25	128
2	25	33	4	30	186
2	30	43			

TABLE 3. ACCELERATION AND CAROTID ARTERIAL PRESSURE DEFICIENCY AREAS

$g \times t_g = \text{constant}$; dog: 43 tests

g	TIME OF EXPOSURE	$g \times t_g$	A.P. DEFICIENCY AREAS
	sec.		$p t_p$
3	16	48	1,065
4	12	48	1,054
5	9.5	48	1,059
6	8	48	1,135

TABLE 4. COMPARISON OF ARTERIAL PRESSURE AREAL CHANGES IN DOG AND MONKEY UNDER POSITIVE AND NEGATIVE g FORCES
Exposure time, 10 sec.

ACCELERATION	ANIMAL	NO. TESTS	ARTERIAL PRESSURE AREA CHANGE		CAROTID-FEMORAL DIFFERENCE
			Carotid Artery	Femoral Artery	
g			pt_p	pt_p	
+1	Dog	178	-128	+ 68	-60
+2	"		-274	+242	-32
+3	"		-438	+358	-80
+1	Monkey	118	- 92	+272	+180
+2	"		-191	+345	+154
+3	"		-378	+542	+164
-1	Dog	162	+ 84	-200	-116
-2	"		+126	-382	-256
-3	"		+406	-512	-106
-1	Monkey	183	+208	- 66	+142
-2	"		+349	-142	+207
-3	"		+578	-265	+313

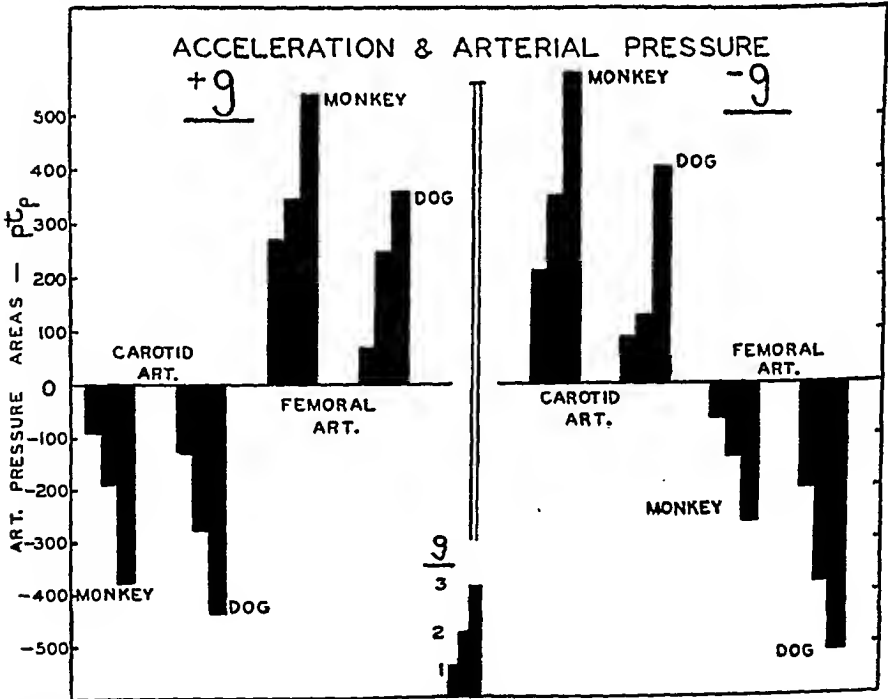


FIG. 5. CHANGES IN CAROTID AND FEMORAL ARTERIAL PRESSURES (areas) in the dog and monkey under exposure to positive and negative accelerations.

and the pt_p deficiency areas calculated. The study revealed clearly that for a given gt_g value, the resultant pt_p areas were a constant (table 3).

Positive and negative g effects compared. An analysis of arterial pressure areal changes shown by animals under positive and negative g forces also indicated striking differences in response of the monkey and dog. Overall changes in the carotid and femoral arterial pressures have been reported in these cases (4). The arterial pressure areal changes have now been determined from over 600 tests, and the data are shown in table 4.

It is striking that in the case of the monkey compared to the dog there is less fall in the carotid and greater rise in the femoral pressure under positive g, and greater rise in the carotid and less fall in the femoral under negative g forces. In comparison with the overall arterial pressure deficiency observed in the dog, the monkey shows a considerable 'credit balance' in arterial pressure under both positive and negative accelerations. Further, and perhaps more important, circulation through the cerebral tissues appears to be better maintained in the anthropoid animal under stress than in the case of the dog (table 4, fig. 5).

DISCUSSION

In animals exposed to acceleratory forces, carotid arterial pressure follows a pattern which is expressed diagrammatically in figure 1, derived from several hundred experiments. After the main fall in arterial pressure (A), there occurred toward the end of the exposure a rather rapid increase in level (B), until a post-centrifugal pressure peak (C) was reached which was often higher than the initial or basal level. This peak was followed by a fall in arterial pressure (D) below the initial pre-test value, then a gradual climb toward the normal level (E). The post-centrifugal fall in arterial pressure may be a reflection of the transient functional disturbance caused by exposure to g forces.

In the dog the time interval $t_p - t_g$ (table 1) was dependent on the intensity of g; in the case of the monkey, on the other hand, return to normal pressure levels following centrifugation was practically independent of the intensity of the exposure up to 4 g. This observation is probably of importance in differentiating between the effect of acceleratory forces on quadruped (dog) and upright (monkey) mammalian forms. The monkey and man as well, perhaps through vascular adjustments (reflexes, etc.) developed in the course of evolution to support the upright posture, are more resistant than lower forms to gravitational forces which may tend seriously to reduce blood pressure in the head end of the animal.

Data on the reactions of man on the centrifuge have been derived from figures published by Wood *et al.* (5) and are included in figure 4 herewith². It is clear that the resistance of man to acceleratory forces is equally as good as that shown by *Macacus rhesus*, on the basis at least of arterial pressure reduction in the carotid vessel.

It is essential to consider the various factors involved in relation to one another rather than separately. Two of the variables, g and t_g , are independent; the others, degree and duration of blood pressure reduction, are dependent. It is

² Acknowledgement is made of the permission of the Mayo Aeromedical Unit to compare these results.

the area gt_g which determines the area of arterial pressure change, pt_p ; i.e., it does not matter how, for example, 100 gt_g units have been produced—they may have been produced by a short exposure to high g or a longer exposure to lower g —provided the gt_g areas are the same (within limits), the resultant pt_p areas will be similar. For this reason the simple minimal level of blood pressure alone does not fully express the physiological difficulty during and responses after centrifugation. Earlier work from this laboratory also suggests that mortality following exposure to centrifugation is a direct function of the product of time and intensity of exposure to g (1).

SUMMARY

The reduction in carotid arterial pressure observed during exposure to acceleratory forces (monkey, dog; 1–4 g , 5–30 sec.) may be considered as a blood pressure deficiency area (pt_p). Thus, one may determine the carotid arterial pressure deficiency as a function of time. This area was found to be directly proportional to the intensity and duration of centrifugation (gt_g), and for a given acceleratory area the circulatory deficiency area was a constant.

Monkeys were found to be much more resistant to acceleration than dogs. In the monkey the arterial pressure deficiency areas on exposure to 1, 2 and 4 g were only about half as great as those in the dog; further, the recovery areas (rebound) were marked in the former and usually absent in the latter case. Carotid arterial pressure reactions in man are similar to those of the *Macaque*.

A comparison of dog and monkey responses under positive and negative g forces also indicated that circulation through the cerebral tissues was maintained better in the anthropoid type. The differences in response may be referable to the different stance and thus the reflex development of the two animal types.

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EFFECT OF SYMPATHECTOMY ON BLOOD FLOW IN THE HUMAN LIMB

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Received for publication January 1, 1948

Clinical papers (1, 2) testify to the increasing frequency with which sympathetic block and resection are being used in the treatment of the peripheral vascular diseases, particularly those in which intermittent claudication is the chief manifestation.

Herrick, Essex and Baldes (3) showed that the blood flow in the femoral artery in the sympathectomized limb of the dog was approximately double that of the normal side. They measured total blood flow which does not distinguish between the circulatory requirements of skin and muscle. Grant and Pearson (4) pointed out the difference in behavior of the circulation in the distal and proximal parts of a human limb. The former, a measure of the skin circulation, responds to sensory stimuli and to adrenalin by vasoconstriction; the latter, the bulk of which is composed of skeletal muscle, responds to sensory stimuli not at all and to adrenalin by vasodilatation. They also showed that there was no difference in the hyperemia of exercise in a forearm deprived of its sympathetic innervation and its normal mate. Since then others, notably Wilkins and Eichna (5), have commented upon the lack of effect of sympathectomy upon muscle blood flow and have even intimated that such an operation might be harmful because vasodilator fibers also are sectioned. This part of their argument presupposes that such a system of nerve fibers actually exists.

Our approach in clarifying the problem is based on a comparison of the blood flow in sympathectomized and normally innervated human limbs.

METHOD

Blood flow was measured by the venous occlusion method (6) which although indirect is reliable and readily applicable to the unanesthetized human. A water plethysmograph of the Abramson type (7) was used for the calf; for the foot, the apparatus described by Kunkel and Stead (8) was more satisfactory.

Eleven of the 12 patients comprising this report had vascular disease in the lower limbs of varying degree and etiology. The remaining individual had persistent hypertension for which the Smithwick operation was done. The highlights of their clinical histories are incorporated in the tables.

The interval between operation and our studies was as short as a week in some instances, while in others it was from six months to several years. In four acute experiments, the test limb also served as control, inasmuch as comparable blood

¹ Aided by a grant from the Sigmund M. Lehman Fund at the Montefiore Hospital, New York City, and the Martha Hall Foundation.

flow records were taken before and after operation. In the others, the contralateral limb served as control or the 'normal' for the individual. Such a comparison was felt to be valid as intensive clinical examination of the peripheral vessels disclosed no striking difference between the two limbs.

Although the segmental distribution of the autonomic nervous system is still incompletely known in some respects, interruption of the ganglia at the level of L2 and L3 has been shown to produce effect from the level of the knee down (9), including the calf muscles. As a check on the adequacy of operation, determination of the rise in skin temperature and of changes in the electrical resistance of the skin was made. These tests as well as the histological report on material removed at operation enabled us to state unequivocally that a sympathectomy had been done, that it was complete and that it included the test region.

Tests were run with the patient seated comfortably in a chair with the leg outstretched and parallel to the ground. When blood flow to the calf was measured, the circulation to the foot was eliminated by a tourniquet applied just distal to the plethysmograph.

An indifferent water temperature of 34 to 35°C. was maintained on the enclosed segment of limb for 30 minutes. The room temperature was constant for each experiment but varied between 23°C. maintained during the winter season and 30°C. during the summer. This is of little importance for circulation of the calf in which we were especially interested. We have confirmed Grant's observation (4) that the blood flow of resting muscle is remarkably constant even though there are such variations in the environmental temperature. However, in the case of the hand or foot, even small temperature changes may profoundly affect the blood supply.

Blood flows of the arbitrarily defined skin and muscle circulation were taken in the resting state described above. This was the average of several test runs which as a rule differed so little that the inflow curves were almost parallel. It was felt that only variation of 20 per cent or more in successive blood flow determinations could be regarded as significant.

Measurements were also taken during the phase of reactive hyperemia. Upon occluding the arterial circulation for ten minutes and then releasing it, a profound increase in blood flow as well as amplitude of the pulse wave was registered on the tracing. The characteristic flush was easily visible in the portions of the extremity outside the plethysmograph. It is our belief that the blood flow during reactive hyperemia is an index of the available peripheral circulation.

In certain instances blood flow estimations were made with the limb segment exposed to water of 45°C. and after exercise of the calf muscles consisting of 50 alternate flexions and extensions at the ankle.

RESULTS

1. *Blood flow in the foot (table 1).* The skin of a recently sympathectomized limb is warm, dry and bright in color. Although these findings tend to persist, there is sometimes a return of skin temperature to preoperative levels in a matter of weeks. Loss of sweating may then be the sole objective sign of a technically complete denervation and may also be partly responsible for making the sympathectomized limb feel warm.

With the rise in skin temperature there is an increase in blood flow. Its magnitude is dependent on two factors: the available vascular bed and the level of the resting blood flow prior to operation.

The maximum increase in blood flow on the sixth day and again three weeks after operation is only 10 per cent in P. Ga., age 21 years, whose basic disease was thrombo-angitis obliterans. This is well within the limit of error. It must be mentioned that this patient had a gangrenous ulcer on the little toe of the test foot with an inflammatory response in the surrounding tissue, so that vasodilatation was almost complete before operation. Thus the resting blood flow pre-operatively approximates the flow during the phase of reactive hyperemia and this accounts for the negligible increase.

In J. R., age 36, who also had Buerger's disease, the blood flow in the test foot increased 85 per cent over its preoperative level on the eighth day.

TABLE 1. BLOOD FLOW IN FOOT (cc/min/100 grams of tissue)

SUBJECT	AGE	RESTING BLOOD FLOW		REACTIVE HYPEREMIA (BLOOD FLOW)	% CHANGE AFTER SYMPATHECTOMY
		Control	Sympathectomy		
P. Ga. ¹	21	5.4	1) 5.6 2) 6.3	7.6	+ 10
J. R. ¹	36	3.5	6.4	8.0	+ 85
R. M. ¹	38	3.8	5.6	10.0	+ 45
J. K. ¹	56	2.8	6.0	20.0	+115
C. J.	45	3.0	5.9	10.0	+ 95

¹ Same foot used.

Patient R. M., age 38, had essential hypertension. Two weeks after the first stage of the Smithwick procedure, the increase is only 45 per cent in spite of the fact that her peripheral vessels were clinically normal. It is known that the extensive circulatory readjustments that follow such a procedure militate against marked vasodilatation in the extremities.

Our fourth patient, J. K., age 60 years, had a history of cold injury which resulted in amputation of several toes for gangrene. His feet, which were extremely cold and sweaty prior to operation, became warm and dry. The increase in blood flow ten days after sympathectomy is 115 per cent.

Patient C. J., age 45 years, illustrates the long-term effects of sympathectomy in the foot. In this individual, where the underlying disease was again thrombo-angitis obliterans, the blood flow in the sympathectomized foot six months after operation is 95 per cent greater than in the 'control' foot.

Inasmuch as sympathectomy for peripheral vascular disease was seldom performed unless there was an adequate rise of skin temperature after paravertebral novocaine block, this automatically excluded from our series most patients with a markedly deficient vascular supply.

2. *Blood flow in the calf.* The resting blood flow of a muscular region like the calf, as determined by the technique employed, ranges from 1.5 to 3.0 cc. per minute per 100 grams of tissue but is fairly constant for the same individual. It has been calculated by Grant (4) that the intense cutaneous hyperemia produced by multiple puncture of histamine into the skin results in an increase of

one to two cc. of blood flow per minute per 100 grams of tissue. This figure represents the increase due to complete vasodilatation in the skin and only measures which produce more than this, which is roughly an increase of 100 per cent, can be considered as affecting muscle blood flow.

It is quite striking, therefore, that in the 12 patients in whom the sympathetic outflow was resected there was no such significant increase.

In contrast, the increase in blood flow during the phase of reactive hyperemia

TABLE 2. BLOOD FLOW IN CALF (cc/min/100 grams of tissue)

SUBJECT	AGE	DISEASE	TIME SINCE OPERATION	RESTING BLOOD FLOW		REACTIVE HYPEREMIA (BLOOD FLOW)	% CHANGE AFTER SYMPATHECTOMY
				Control	Sympathectomy		
P. Ga. ¹	21	TAO ²	1) 6 days 2) 21 days	2.7	1) 2.1 2) 2.3	19	-20
J. R. ¹	36	TAO	8 days	2.1	1.6	15	-25
R. M. ¹	38	Essential hypertension	14 days	1.9	1.9	10	0
J. K. ¹	56	Cold injury	8 days	2.7	3.5	18	+30
C. J.	45	TAO	6 months	1.5	2.9	13	+9
J. McL.	60	Cold injury AS ³	15 days	1.2	1.2	8.2	0
T. N.	63	AS	1 year	Bilateral lumbar sympathectomy	Left) 2.2 Right) 2.1	2.3 4.5	—
E. B.	60	AS	14 days	2.6	3.7	9.5	+40
P. GI.	38	TAO	4 months	2.3	2.3	14	0
F. C.	44	Raynaud's syndrome	2½ years	Quadrilateral sympathectomy	2.8	36	—
L. G.	33	Diffuse vascular disease	3 years	Bilateral lumbar sympathectomy	3.4	10	—
W. F.	67	AS	15 days	Unilateral amputation	2.9	11.2	—

¹ Same calf used.

² Thromboangiitis obliterans.

³ Arteriosclerosis.

may be as much as 300 to 1200 per cent of the resting value. Less marked but also significant is the increase which results from moderate exercise of the calf muscles or from heating the segment of calf enclosed in the plethysmograph.

The increase in blood flow to any of these measures is limited by the available vascular bed. Thus, in a younger patient like P. Ga., this potential reservoir is much greater than in an older individual, T. N., whose pain at rest indicates that the tissues are insufficiently supplied with blood even under basal condition.

DISCUSSION

This study shows that sympathetic denervation of the human limb results in an increased blood flow in areas where skin predominates but leaves the blood flow in muscular regions relatively unchanged. Any augmentation noted in the latter can easily be explained by the coexisting cutaneous vasodilatation. Even in sympathectomized regions where skin circulation predominates, the

relation of resting flow to the blood flow during reactive hyperemia shows that maximal vasodilatation is not present within a week after operation.

In man, vasoconstriction of the skin, an increase in pilomotor and sudomotor activity and vascular dilatation in muscle are the proven effects of sympathetic stimulation and adrenalin discharge on the limbs (4, 10, 11). Conversely, resection of the sympathetic pathways leads to cutaneous vasodilatation and loss of sweating and hair erection in the involved region. Our results bear out the contention arrived at from the intra-arterial injection of adrenalin: that muscle vessels would not be dilated by the removal of sympathetic control.

The vascular bed of a muscular region can be opened wide by exercise, by direct heating of the limb and by creating a blood deficit through occlusion of the main artery. Each of these measures produces a significant increase in blood flow through the release of vasodilating metabolites. Similar increases take place despite the presence of definite vascular disease, provided the collateral circulation is adequate. The constant values for blood flow in muscle after sympathectomy permit the conclusion that innervation of blood vessels is not the important factor in regulating the circulation in muscular tissue. This favors for skeletal muscle an assumption which has been previously made for the brain (12) and cardiac muscle (13); namely, that it is the local demand which decides the blood flow to the active area.

Comparing the vascular reactions of blood vessels in normal muscle with those in the muscle of patients with vascular disease may seem unwarranted. There is no proof, however, that there is an essential difference in their behavior to sympathectomy. The circulation in areas where skin predominates, such as the hand and foot, is increased after sympathectomy both in the normal state and in the peripheral arterial diseases. Such an increase is quantitatively less striking in the latter, because of a diminution of the vascular bed through organic obstruction of blood vessels and sometimes because maximal vasodilation is reached as a result of secondary inflammation or due to compensatory circulation developed in response to the local ischemia.

From a clinical viewpoint, important inferences may be drawn. 1) Exercise, direct heating or anoxia of tissue increase muscle blood flow, but a sympathectomy is relatively impotent in this respect. The first two procedures actively increase the metabolism of the limb, while in the third the blood deficit is rapidly repaid on release. Each of these modalities, somewhat modified, is used in the treatment of peripheral vascular disease (reflex vasodilatation, intermittent venous occlusion); each has practical disadvantages which curtail its use. The ideal measure is one which would cause vasodilatation without increasing the metabolism of ischemic tissues.

2. Resection of the sympathetic trunk in an attempt to increase blood flow and thereby reduce the pain of muscular ischemia, or to increase the functional capacity for exercise or as a prophylactic against the reduction of muscle circulation by the occlusive vascular diseases, appears to be of doubtful value.

3. The use of sympathectomy for effect on the skin or lesion of the hand or foot is logical and sound physiologically. Thus in hyperhidrosis of severe degree, in disfigurement due to marmorization of the skin, and more frequently in Raynaud's phenomena and in the causalgic state, there is such marked relief both subjectively and objectively that the procedure seems justified. Ulcera-

tions which have been intractable to other treatment frequently heal. On occasions we have seen beginning gangrenous lesions of the toes slough and clear up promptly following sympathectomy. We have also seen toes which merely appeared poorly nourished and cyanotic become gangrenous immediately after operation. It is reasonable to assume that blood had been diverted from the local area which needed it desperately to a large skin surface with resultant detrimental effect on the area for whose benefit the sympathectomy was originally planned.

Furthermore, there are large gaps in our knowledge of the autonomic nervous system, not only as regards function but also anatomical distribution. Some evidence is accumulating for the existence of vasodilator fibers within the sympathetic system in man (14). If the sympathetic trunk is severed, such fibers which might play a beneficial rôle are removed with the vasoconstrictor fibers. On the other hand, if the sympathetic pathways carry afferent impulses, their interruption might very well be warranted for relief of pain, whether due to intermittent claudication or other factors. A recent article on afferent conduction by Threadgill merits attention in this respect (15). However, we should not at the present time base the need for operation on these hypotheses but on the more solid foundation of effect on muscle or skin vessels.

SUMMARY

1. A lumbar sympathectomy which is effective when gauged by the changes produced in an area of predominant skin circulation (foot) produces minimal increase in the circulation of a predominantly muscular area (calf).

2. The effective stimulus in increasing muscle blood flow is one which releases vasodilating metabolites. Exercise, direct tissue heating and arterial occlusion and release are examples.

3. The clinical application of sympathectomy for lesions or symptoms in areas primarily of skin circulation and other areas which are composed chiefly of muscle are discussed. The use of sympathectomy for the latter is of doubtful value.

We are indebted to Dr. Louis Leiter, Chief of the Medical Division, Montefiore Hospital, for advice and criticism and to Mr. Abraham Seigel for his valuable technical assistance.

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EFFECTS OF EXERCISE AND OF SOME OTHER INFLUENCES ON THE RENAL CIRCULATION IN MAN¹

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Received for publication January 29, 1948

Relatively few quantitative reports are available on reflex influences on the renal circulation in man. Smith (1, 2) found that both diodrast and inulin clearances fell on changing from recumbent to upright or near upright position; filtration fraction showed slight rise or no change. McCann and Romansky (3) found that on changing from recumbent to erect position patients with nephrop-tosis showed falls in diodrast clearance with no significant change in inulin clearance, the filtration fraction rising; normal subjects and hypertensives with-out nephrop-tosis showed no significant postural changes in clearances or in filtra-tion fraction. Brun, Knudsen and Raaschou (4) confirmed the findings of many earlier workers that urine flow is lower in the erect (or 60° vertical) position than in recumbency. Inulin clearance falls in the vertical position, with an increase in the percentage of filtered water reabsorbed. Diodrast clearance falls more than inulin clearance, giving a rise in filtration fraction. If the erect position is maintained to the occurrence of syncope, diodrast and inulin clearances and urine flow fall abruptly (1, 2, 5); there is evidence that increased secretion of pituitrin may contribute to the postsyncopal oliguria (5).

Smith (1, 2) found a marked fall in diodrast clearance with marked rise in filtration fraction when subjects became alarmed. Wolf (6) found that a mildly painful cold stimulus (immersion of one hand in water at 5-10° C.) increased the diodrast and inulin clearances, with no change in filtration fraction; in the post-stimulus period diodrast clearance usually fell with no decrease in inulin clearance, giving a great increase in filtration fraction. More severe pain, by pressure on head, reduced both diodrast and inulin clearances, with rise in filtration fraction. There is great variability in Wolf's findings.

Diodrast clearance decreased by 18 to 54 per cent as a result of running 440 yards at maximum speed, and remained below the pre-exercise level for 10 to 40 minutes after exercise. Inulin clearance fall was less than diodrast clearance fall and might not occur. Exercise for 15 minutes on a bicycle ergometer also reduced clearances. There was no correlation between changes in clearances and in urine flow (7) and no mention is made of proteinuria.

This paper reports on the effects of postural changes, of a cold stimulus, of exercise, and of increased protein intake on para-aminohippurate (PAH) and inulin plasma clearances and, in some experiments, on plasma glucose levels, on occurrence of proteinuria, on blood pressure and on renal vascular resistance.

¹ Aided by a grant from the Commonwealth Fund.

Urine volumes were also followed, but water intake was not closely enough controlled to permit other than gross conclusions as to the effects on urine flow. The subjects were healthy males, all except one (*HLW*, age 51) being medical students in the age group 20 to 30 years.

METHODS

Except for the experiments with high protein intake, the subjects had a light breakfast and omitted lunch on the days of experiments, which began about 2:00 P.M. Urine flow was promoted by drinking one to two liters of water during the 60 to 90 minutes before the beginning of the first clearance period. The subjects were recumbent for 30 to 40 minutes before the clearance periods began, the first two of which were controls in recumbency. Clearance periods, except for some shorter periods of running, were about 20 minutes. A priming intravenous dose of 19 mgm. inulin per kilo was followed by a sustaining subcutaneous dose of 90 mgm. inulin and 30 mgm. PAH per kilo. The intravenous inulin was the 10 per cent solution of U. S. Standard Products Co., Woodworth, Wis. The subcutaneous injection was prepared by boiling the necessary amount (0.9 cc. per kilo) of the 10 per cent inulin to about one-third its original volume and adding the 20 per cent PAH solution² (0.15 cc. per kilo). Thus, an 80 kilo-gram subject received 15.2 cc. of 10 per cent inulin intravenously, followed by a mixture of 24 cc. of 30 per cent inulin and 12 cc. of 20 per cent PAH subcutaneously. The 20 per cent PAH is too irritating to be injected undiluted subcutaneously. The subcutaneous injection was given in two or three post-axillary sites and the needle moved two or three times for each skin puncture, so that only a few cc. were given in one region. No anesthetic was used. The first clearance period was begun 20 to 25 minutes after ending the subcutaneous injection. This method obviated the necessity of continuous intravenous infusion and permitted the subject to move about freely for any desired experimental procedure (8). A few representative plasma level curves are given in figure 1; mean plasma levels during the respective clearance periods are calculated from such curves.

Urine collections were by voluntary emptying of the bladder. Some days prior to an experiment the subject obtained a water diuresis curve; smoothness of the curve was taken to indicate satisfactory sample collections. We are convinced that the majority of normal, intelligent, co-operative human males can quickly learn to empty the bladder quantitatively, and that a smooth water diuresis curve is a good index of such ability. Blood samples were heparinized and centrifuged immediately after collection. Blood pressures were obtained frequently by the auscultatory method; mean pressure was taken as diastolic plus 40 per cent of pulse pressure and the values given in the tables are the averages of values during a corresponding clearance period. Renal resistance units per square meter (RRU/M^2) are expressed as

² The PAH solution was generously furnished by Sharpe and Dohme Co., Philadelphia, Pa.

$$\frac{\text{mean arterial pressure (mm. Hg) minus 5.}}{\text{renal blood flow per sec. per M}^2}$$

Renal blood flow was taken as twice the PAH plasma clearance; errors due to hematocrit variations were neglected, since we were interested only in the grosser directional changes in renal vascular resistance. Filtration fraction (FF) is $\frac{\text{inulin clearance}}{\text{PAH clearance}}$. The zero settings of the photoelectric colorimeter for

the plasma PAH and the plasma inulin determinations were made on pre-injection blank plasma filtrates; correction was made for changes in color production in the plasma inulin procedure due to changes in plasma glucose.

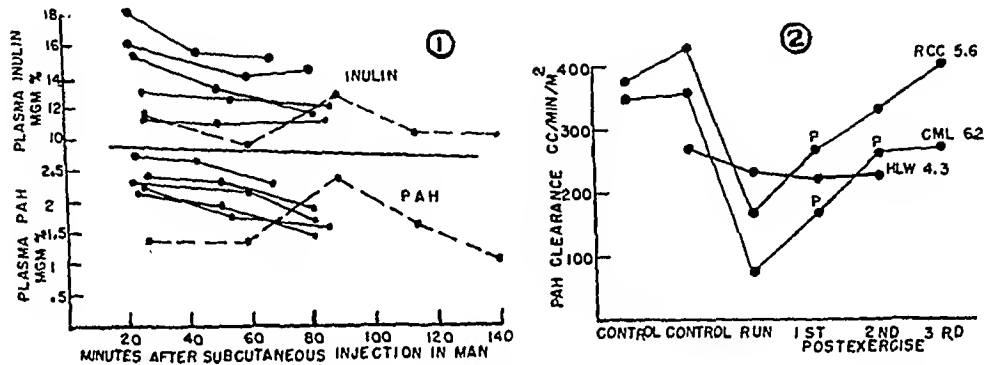


FIG. 1. INULIN AND PAH PLASMA LEVELS following intravenous priming and subcutaneous sustaining injections used in these experiments. The solid line separates the inulin from the PAH curves. The broken curves are from an exercise experiment; the first two points are during rest, while the third points are higher because of the diminished rate of excretion during exercise between second and third samples.

FIG. 2. PAH CLEARANCES BEFORE, DURING AND AFTER EXERCISE on subjects, *HLW*, *RCC*, and *CML*. Speed of running in miles per hour at ends of curves. Presence of protein in urine samples is indicated by P.

Effect of high protein intake. Control periods in recumbency six hours after a light breakfast were obtained in two subjects on uncontrolled diet containing about 90 grams of protein daily. Some days later clearances were again obtained in recumbency four hours after a large protein meal, consisting largely of beef steak. On one subject, *HW*, clearances were again obtained after a week of high protein diet (220 grams of protein with 3400 calories daily). The results are seen in table 1. Increase in protein intake as a single meal had no effect on PAH clearance or on renal vascular resistance but slightly raised inulin clearance; high protein intake for a week raised PAH clearance 18 per cent and inulin clearance 31 per cent, and lowered renal vascular resistance 12 per cent.

Postural effects. Five experiments on the effects of postural changes were carried out on five subjects; the results are seen in table 2. The PAH clearance in the standing period was 12, 24, 15 and 23 per cent lower than the average of the two lying periods for the first four experiments of table 2, averaging 19 per cent

lower. The inulin clearance in the standing period was 1, 18, 8 and 6 per cent lower than the average of the two lying periods, averaging 8 per cent lower. The inulin clearance fall on standing may be compared with an earlier finding (9) that endogenous creatinine output is 9 per cent lower on standing than in recumbency. In the earlier work clearances cannot be calculated since no plasma determinations were made but, since the plasma creatinine levels remained constant, the rates of urinary output are proportional to the clearances. Subject RO showed some vasomotor disturbance at the time of the subcutaneous injection, losing consciousness for a brief period. It is believed that the clear-

TABLE 1. EFFECT OF HIGH PROTEIN INTAKE ON CLEARANCES

SUBJECT DATE	URINE FLOW	PAH CLEARANCE	INULIN CLEARANCE	F.F.	PLASMA GLUCOSE	MEAN B.P.	RRU/M ²
	cc/min/M ²	cc/min/M ²	cc/min/M ²		mgm. %	mm. Hg	
Uncontrolled diet (about 90 grams protein)							
HW 9/24/47	0.59	350	59	0.169		88	7.1
	0.82	395	62	0.157		84	6.0
4 hours after large protein meal							
HW 11/4/47	6.22	380	67	0.176	73	88	6.6
	2.71	376	66	0.176	77	88	6.6
	1.81	373	51	0.137	77	87	6.6
Protein intake 220 grams daily for week preceding 1/22/48							
HW 1/22/48	1.18	454	83	0.182	95	89	5.6
	1.08	427	72	0.168	75	89	5.9
	1.04	442	84	0.190	79	88	5.8
Uncontrolled diet (about 90 grams protein)							
FC 10/15/47	1.15	335	58	0.172	88	89	7.5
	4.27	339	59	0.174	86	90	7.5
4 hours after large protein meal							
FC 11/11/47	0.56	341	66	0.194	105	90	7.5
	0.63	333	67	0.20	94	91	7.7

ance values of his first period are abnormally low, due to renal vasoconstriction as a result of the psychic reaction to the injection; the second control period should be taken as the recumbent control with which to compare the standing period. When this is done there is seen on standing a fall of 23 per cent in PAH and a rise of 10 per cent in inulin clearance, the filtration fraction rising from 0.162 to 0.231. Thus in all cases the filtration fraction was higher during standing, the inulin clearance usually showing a slight fall but less than the PAH. In three of four cases mean blood pressure was slightly higher on standing but there was no great change. Since cardiac output is lower on standing than on lying, total peripheral resistance must be somewhat increased on standing. That the renal vascular bed participates in this constriction is shown by the fact

that in every case renal vascular resistance is higher on standing than on lying. A single observation of a postural change in renal vascular resistance of these magnitudes would not be significant, but it is believed that the consistency of behavior justifies the view that the usual normal response on changing from the recumbent to the standing position is a slight increase in renal resistance, due to vasoconstrictor impulses rather than to adrenalin output.

Effects of cold. Three experiments were performed on three subjects; one of these has been omitted because great fluctuations in the control periods indicated that urine collections were not quantitative. All periods were in recumbency, the first two being controls, while cold was applied during the third period.

TABLE 2. POSTURAL EFFECTS ON CLEARANCES

SUBJECT DATE	POSTURE	URINE FLOW	PAH CLEARANCE	INULIN CLEARANCE	F.F.	PLASMA GLUCOSE	MEAN B.P.	RRU/M ²
		cc/min/M ²	cc/min/M ²	cc/min/M ²		mgm. %	mm. Hg	
HLW 5/1/47	lying	4.33	259	47.8	0.185			
	lying	4.87	258	46.4	0.180			
	standing	3.66	228	46.5	0.204			
HW 9/24/47	lying	0.59	350	59.0	0.169		88	7.1
	lying	0.82	395	61.8	0.157		84	6.0
	standing	0.73	283	48.8	0.173		92	9.2
RJP 10/1/47	lying	0.94	387	74.7	0.193		94	6.9
	standing	2.13	304	61.5	0.202		96	9.0
	lying	0.78	318	59.1	0.186		94	8.4
FC 10/15/47	lying	1.15	335	58	0.172	88	89	7.5
	lying	4.27	339	59	0.174	86	90	7.5
	standing	2.9	261	55	0.211	88	85	9.2
RO 10/8/47	lying	0.21	219	41	0.185		85	5.9
	lying	0.24	319	52	0.162		86	4.1
	standing	0.25	245	57	0.231		92	5.7

With *HLW* the cold stimulus was applied by immersing a hand and about a third of the forearm in a mixture of ice and water at 0° C. The immersion was not continuous, since the procedure was too painful. Immersion was continued for a few minutes until the pain became intense; the hand was then withdrawn for a minute and the cycle repeated. In order to avoid the pain element, *subject FAH* dipped a hand and lower forearm into water at 14–15° C. To avoid the complication of adaptation, every three minutes the hand was removed and the other hand immersed, while the nonimmersed hand was dried and warmed by body contact; the cycle was repeated throughout the third clearance period. It is seen in table 3 that *HLW* (cold plus pain) showed a slight fall in both clearances, a questionable rise in filtration fraction, and a significant rise in mean blood pressure and renal vascular resistance. *Subject FAH* (cold alone) showed

no change in any of the values. It is quite probable that exposure of a sufficiently large area of body surface to a temperature of 14–15° C. would result in significant renal vasoconstriction, but the threshold is obviously higher than that of the hand and forearm, since application of a very slight cold stimulus (evaporation of a few drops of ether) to one hand in a large series of student experiments has consistently produced striking vasoconstriction in the other hand.

Exercise. Eight experiments have been performed on six subjects; two of these are omitted because of great fluctuations, presumably due to inadequacy of urine collections, in the control periods. The results of the six satisfactory experiments are seen in table 4. All periods except the running were in recumbency. Blood pressures were taken at three- or four-minute intervals except during the period of running; they were not taken in the experiment on *HLW*. Blood pressure readings were not begun after exercise until the subject had emptied his bladder; the first postexercise reading was usually two or three

TABLE 3. EFFECTS OF COLD ON RENAL CIRCULATION

SUBJECT DATE	PROCEDURE	URINE FLOW	PAH CLEARANCE	INULIN CLEARANCE	F.F.	MEAN B.P.	RRU/M ²
		cc/min/M ²	cc/min/M ²	cc/min/M ²		mm. Hg	
HLW 6/9/47	control	1.12	257	49.5	0.193	102	11.3
	control	3.45	247	47.8	0.194	98	11.3
	cold	4.75	221	44.5	0.201	113	14.7
FAH 10/29/47	control	0.56	373	63	0.168	90	6.9
	control	0.38	375	61	0.163	91	6.9
	cold	0.52	375	62	0.166	93	7.0

minutes after exercise had stopped. On this reading the systolic pressure usually was higher and the diastolic lower than before exercise; the systolic then fell and the diastolic rose. Individual readings are not shown in the table; mean blood pressure indicates the average of the mean pressure values taken during a given period. Mean pressures during the periods after exercise have always been slightly lower than the pre-exercise control values; total peripheral resistance thus remains lowered for some time after exercise. Mean arterial pressure during exercise was not determined, but is known to be at least as high as at rest. The values for RRU/M² during exercise are given as minimum values, taking mean pressure during exercise as being the same as at rest.

During light exercise (*HLW*) PAH and inulin clearances fell slightly and remained down for at least 40 minutes after exercise; the urine remained protein-free as tested by heat. During moderate to moderately severe exercise (*LHR*, 12/18/47 and *RCC*) both clearances fell; the return of PAH clearance toward normal may or may not be completed at the end of 50 to 60 minutes, the return of inulin clearance to normal being faster. Behavior of filtration fraction during and after exercise has not been consistent. During exercise of this degree renal

TABLE 4. EFFECTS OF EXERCISE ON RENAL CIRCULATION

SUBJECT DATE	PROCEDURE	URINE FLOW	PAH CLEARANCE	INULIN CLEARANCE	F.F.	URINE ALBUMIN	PLASMA GLUCOSE	MEAN B.P.	RRU/M ²
		cc/min/M ²	cc/min/M ²	cc/min/M ²			mgm. %	mm. Hg	
HLW 3/11/47	During 13 of the 15 minutes of second period subject jogged at 4.3 miles per hour. Respiration and pulse rate increased, slight sweating, not dyspneic or exhausted.								
	lying	1.34	266	54	0.205	—			
	running	1.17	232	50	0.214	—			
	lying	1.34	222	47	0.213	—			
	lying	1.25	224	45	0.201	—			
CML 11/12/47	During 12 of the 14 minutes of third period subject ran at 6.2 miles per hour. Dyspneic, exhausted, sweating profusely.								
	lying	5.25	343	67	0.197	—	89	97	8.1
	lying	5.65	357	63	0.177	—	87	96	7.7
	running	1.60	74	17.6	0.237	—	112		37+
	lying	0.37	164	31.2	0.190	++		88	15.2
	lying	0.97	260	33.6	0.129	+	98	90	9.8
	lying	0.47	264	34	0.128	—	87	90	9.7
RCC 11/19/47	During 11 of the 15 minutes of third period subject ran at 5.6 miles per hour. Slight dyspnea, not exhausted, moderate sweating.								
	lying	0.52	375	59	0.157	—	98	97	7.4
	lying	0.53	429	56	0.131	—	97	94	6.2
	running	0.16	168	22	0.132	—	159		16+
	lying	0.87	267	46	0.174	+		86	9.1
	lying	0.36	326	57	0.174	—	122	87	7.5
	lying	0.98	396	57	0.145	—	104	85	6.1
LHR 11/18/47	During 11 of the 13½ minutes of third period subject ran at 7.2 miles per hour. Dyspneic, exhausted, sweating profusely.								
	lying	5.1	403	67	0.165	—	92	97	6.8
	lying	6.7	388	66	0.169	—	90	95	7.0
	running	1.73	79	13.6	0.174	—	190		35+
	lying	0.75	179	33	0.185	++		86	13.6
	lying	0.56	230	53	0.232	+	145	92	11.3
	lying	0.38	275	58	0.212	—	122	89	9.2
LHR 12/18/47	During 19½ of the 20½ minutes of the third period subject ran at 5.7 miles per hour. Hyperpnea but not dyspnea, moderate sweating, not exhausted as on 11/18/47.								
	lying	1.57	398	64	0.161	—	86	97	6.9
	lying	4.84	388	60	0.155	—	84	95	7.0
	running	1.67	178	38	0.214	—	100		15+
	lying	3.60	293	67	0.23	—		94	9.1
	lying	5.60	274	61	0.223	—	97	95	9.8
	lying	7.05	276	60	0.217	—	84	95	9.8
LHR 1/8/48	During 20 of the 22 minutes of third period subject ran at 6.7 miles per hour. Dyspnea, sweating profusely, exhaustion almost as great as on 11/18/47.								
	lying	5.8	340	58.6	0.17	—	83	96	8.0
	lying	6.31	357	61.5	0.17	—	83	93	7.4
	running	1.29	123	25.3	0.21	—	108	95+	22+
	lying	1.28	333	65.8	0.20	—		85	7.3
	lying	1.05	311	55.8	0.18	—	94	85	7.8
	lying	2.87	328	54.1	0.17	—	85	87	7.5

vascular resistance at least doubles; it may or may not have returned to normal after 50 to 60 minutes. Plasma glucose is elevated to a variable extent, being back to normal by 60 minutes after exercise stops. The plasma glucose value shown as during exercise was on a sample taken two to four minutes after exercise stopped; the values shown for the fifth periods were at about 30 minutes after exercise stopped and the values for the sixth periods were at about 60 minutes. They are true glucose values on a cadmium or zinc filtrate. Since all samples were venous, they do not represent the full extent of the rise in arterial plasma glucose. Considerable increases in plasma glucose on exercise probably indicate increased output of adrenalin, but this point cannot be regarded as settled.

The speed of running cannot be taken as an absolute index of the severity of the exercise; running 6 miles an hour for 15 minutes is much more of an effort for some subjects than for others. Thus, in the two experiments classified as 'moderate to moderately severe exercise', 5.6 miles an hour for 11 minutes produced somewhat greater subjective effects in *RCC* than did 5.7 miles an hour for 19½ minutes in *LHR*, 12/18/47. The PAH clearance falls were comparable in the two cases, but with *RCC* the fall in inulin clearance and the rise in plasma glucose were greater, and protein appeared in the first postexercise urine sample. Even if the degree of effort could be quantitatively controlled, it seems improbable that all subjects would respond with increased adrenalin output at the same effort level. Thus, one would expect a better correlation between effort level and rise in plasma glucose in a given subject than would be found among different subjects; this seems to be the case, judging from the small number of experiments available here. Taking rise in arterial plasma glucose as a tentative index of adrenalin output, a better approach to an answer should be obtained by carrying out a graded series of exercise experiments on each of several subjects.

The experiments on *CML* and on *LHR*, 11/18/47, are classified as severe exercise, carried practically to the limits of the subjects' capabilities. During the period of exercise both clearances fell markedly and renal vascular resistance increased at least five-fold, the values returning toward normal in the hour after exercise. The question may be raised whether the falls in PAH clearance represent uniform reduction of flow through the various parallel vascular channels or whether there is selective constriction, with some channels being closed. A point of some interest is that in these experiments protein has not appeared in the urine formed *during* exercise but is present in the first one or two approximately 20-minute samples taken after severe (*CML* and *LHR*, 11/18/47) or moderately severe (*RCC*) exercise. If the blood flow were uniformly reduced through all glomeruli, one would expect the proteinuria to appear during the period of greatest ischemia, i.e., during exercise, rather than in the postexercise period when blood flow is improving. This suggests the possibility of differential constriction of various parallel renal vascular channels. If, during heavy exercise, some of the glomeruli have no blood flow or have a capillary pressure too low to permit filtration, the failure of protein to appear in the urine during this period can be explained; when flow and filtration are re-established after exercise, protein appears in the filtrate of the reversibly damaged glomeruli.

Another possibility may be considered. Assume that during exercise there is no differential constriction of parallel channels and that blood flow is reduced to 20 per cent of normal in all glomeruli but that protein does not appear in the urine formed during exercise simply because the period of exercise (11 to 12 minutes, *LHR*, 11/18/47, and *CML*) was not long enough to cause the reduced blood flow to damage the glomeruli sufficiently, and that the process of damage continued into the postexercise period even though blood flow was improving. To investigate this possibility, the experiment of 1/8/48 on *LHR* was carried out. Here the objective was to have the subject exercise to the limit of his capacity for 20 minutes. If protein failed to appear in the urine formed during exercise of 20 minutes while appearing in postexercise samples it probably could not be because of insufficient duration of uniformly reduced glomerular blood flow which still maintained uniformly reduced filtration, but would be more

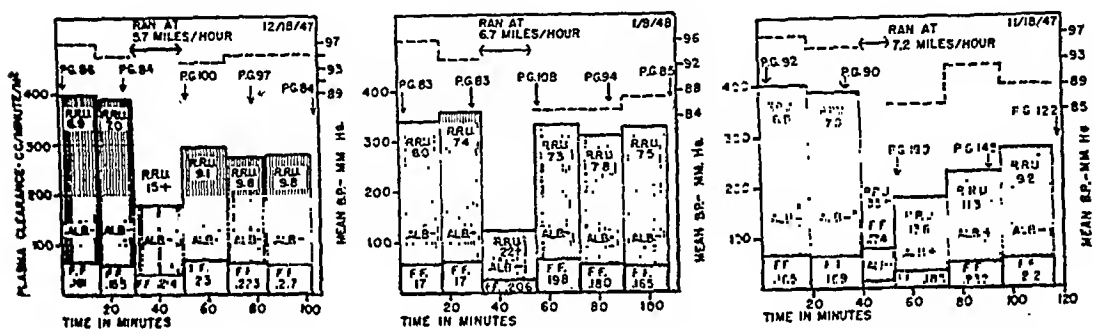


FIG. 3. PAH (HATCHED BLOCKS) AND INULIN (OPEN BLOCKS) CLEARANCES, filtration fractions (F. F.), mean arterial pressure (mean B.P., broken line), renal resistance units per M^2 (RRU), plasma glucose (P.G.) and presence or absence of protein in urine (ALB+ or ALB-) in three exercise experiments on *LHR*. Running at speeds designated in third periods; all other periods in recumbency.

probably explained on the basis of abolition of filtration in the ischemic glomeruli during exercise, the protein appearing as filtration is reestablished after exercise.

The findings on *LHR*, 1/8/48, were unexpected in that even though he ran practically to the limit of speed compatible with 20 minutes' duration, proteinuria did not occur and the clearances fell less than on 11/18/47, where the speed was higher but the duration shorter. The findings of the three experiments on *LHR* are presented in graphic form in figure 3. It appears that for a given subject a certain level of work performance must be reached in order to get maximum fall in clearances and rise in renal vascular resistance and appearance of proteinuria. Work at a lesser rate, even though it be continued longer so that at the end of the period the subject is almost equally exhausted, is less effective. The experiment on *LHR*, 1/8/48, thus fails to answer the question raised in the preceding paragraph, since it is not possible to have a subject work for 20 minutes at the rate which is his maximum for 11 minutes. The experiments on *LHR* show a graded series of responses to a graded series of work rates. This proportionality makes it probable that at work rates below a critical level for a

given subject the response is a vasoconstriction in all the channels, with no ischemic and functionless glomeruli and therefore no postexercise proteinuria, while above this critical level glomeruli begin to drop out of function, in increasing numbers as the work level is increased, so that when glomerular function is re-established after exercise protein appears in the filtrate of these reversibly damaged glomeruli. Significant dropping out of glomeruli does not begin until renal blood flow has first been reduced to below half of the resting value. Since in normal subjects the critical level of work required to cause any significant number of glomeruli to drop out is higher than can be maintained for more than a few minutes, the kidneys are automatically protected against prolonged ischemia during prolonged periods of work.

The border line between the work level for a given subject which is followed by proteinuria and that which is not is quite sharp. Thus, with *LHR*, running at 7.2 miles per hour for 11 minutes brought out protein in the first two post-exercise samples, while 6.7 or 5.7 miles per hour for 20 minutes failed to produce proteinuria. For *CML* (table 4 and figure 2) the effect of running 6.2 miles per hour for 12 minutes was as great as was 7.2 miles per hour for 11 minutes with *LHR*, and *CML* showed protein in the first two postexercise samples. Running at 5.6 miles per hour was somewhat less strenuous for *RCC*, who showed protein only in the first postexercise sample. *Subject LHR* was in the best physical condition, was capable of greater work performance, and required a higher level of performance to bring on proteinuria. The speed of running for a period of a few minutes necessary to bring on proteinuria would probably show good correlation with other criteria of physical fitness, the threshold speed for the less fit being about 5.5 or less and for the most fit 7 or more miles per hour.

These findings thus support, although they do not prove, the view that under great stress many glomeruli may cease functioning, a view recently restated with additional evidence (10). They indicate that such dropping out of glomerular function occurs only under great stress and that the usual degrees of exercise cause a generalized renal vasoconstriction without determinable dropping out of glomeruli. Evidence against glomerular intermittence as a normal occurrence in the anesthetized dog and rabbit has been obtained on the basis of completeness of renal vascular injection with India ink (11). Constancy of glucose Tm indicates absence of glomerular intermittence in man at rest, during diuresis or periods of renal hyperemia, or under the influence of various drugs, although a sufficiently large dose of adrenalin can close many glomeruli (12).

DISCUSSION

It is shown that the renal vascular bed constricts in response to the normal stimuli of exercise and that the degree of constriction is proportional to the severity of exercise, the resistance being increased at least five-fold during heavy exercise. Cutting down of the renal blood flow from a liter or more per minute at rest to 200–250 cc. per minute during exercise contributes about a liter of extra blood flow to the active regions. In so far as a rise in plasma glucose may be taken as an index of adrenalin output, it appears that significant increase in

adrenalin output does not occur until exercise reaches a high level. The renal vasoconstriction seen on light to moderate exercise is apparently due to vasoconstrictor nerve impulses, the constricting action of adrenalin on the renal vessels being added as exercise becomes more severe. The renal vasoconstriction of maximum exercise for relatively brief periods may progress to the point where many glomeruli drop out of function, as indicated by postexercise proteinuria; whether this comes on only when adrenalin output is increased cannot be stated with certainty.

A further possibility, suggested by Trueta's work (10), that during exercise the PAH plasma clearance is no longer a measure of renal plasma flow because of shunting of blood through regions which do not extract PAH, may be considered. According to Trueta, stimulation of the splanchnic nerve or of the renal nerve plexus, or massive intravenous doses of adrenalin may, particularly in the rabbit, cause selective constriction of the cortical vessels to complete or almost complete closure, with diversion of blood through the juxtamedullary glomeruli and the vasa recta springing from their efferent arterioles. Our findings show conclusively that the 'effective' renal plasma flow is reduced but do not tell whether the constriction is confined to the cortical vessels. Nor do they tell whether 'effective' renal plasma flow becomes less than total renal plasma flow, due to diversion of blood into noneffective renal tissue. If Trueta's concept of the diversion channels is correct, some of the diverted blood passes "from the 'arcuate' and proximal parts of the interlobular arteries, via the juxtamedullary glomeruli, to the corresponding veins without traversing a capillary *network*" (p. 64). This would result in less complete PAH extraction and PAH plasma clearance, while still a measure of 'effective' renal plasma flow, would no longer be a measure of total renal plasma flow. Our experiments do not exclude the possibility of diversion through noneffective channels, but since the fall in PAH clearance increases progressively with increasing exercise levels, the results seem more simply explained on the basis of progressive vasoconstriction, without invoking the opening of shunts. A positive answer could be obtained by observing, through renal vein catheterization, whether the renal extraction of PAH is diminished on exercise; such experiments have not been attempted.

SUMMARY

1. Increase in protein intake at a single meal had no significant influence on PAH clearance or on renal vascular resistance in normal man and slightly raised inulin clearance; 220 grams of protein daily for a week raised PAH clearance 18 per cent and inulin clearance 31 per cent, and lowered renal vascular resistance 12 per cent.

2. PAH clearance averaged 19 per cent lower and inulin clearance 8 per cent lower on standing than in recumbency; renal vascular resistance averaged 22 per cent higher on standing, due to vasoconstrictor impulses rather than to increased adrenalin output.

3. Immersion of a hand in painfully cold ice water at 0° C. produced a slight fall in PAH and inulin clearances with a slight rise in mean blood pressure; a pure

cold stimulus through hand immersion in water at 14–15° C. had no effect on clearances or mean blood pressure in the one normal subject studied.

4. Light exercise produces slight falls in PAH and inulin clearances. Moderate to moderately severe exercise lowered PAH and inulin clearances to half or less of their resting values and at least doubled renal vascular resistance; protein may appear in the first postexercise urine samples. Brief maximum exercise lowers PAH and inulin clearances to 20 per cent or less of their resting values while renal vascular resistance is increased at least five-fold; a longer period of maximum exercise at a lower rate is less effective, reducing clearances to about a third of their resting values. Rise in plasma glucose, taken as a tentative index of increased adrenalin output, usually is not marked unless exercise is severe; in a given subject it is proportional to the severity of exercise.

5. Protein did not appear in the urine formed during even the severest exercise of these experiments but after severe exercise was present in the first one or two postexercise samples. The interpretation of the findings is that exercise brings on generalized renal vasoconstriction and that only at the maximum work levels do any large number of glomeruli drop out of function, occurrence of postexercise proteinuria being taken as an index of such dropping out during exercise.

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DURATION OF RENAL ISCHEMIA REQUIRED TO PRODUCE UREMIA¹

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Received for publication February 24, 1948

Bywaters and his colleagues (1) early in the late war drew attention to the fact that shock from crush injuries and other traumata is not infrequently followed by renal failure that persists after other signs of shock have disappeared and can result in fatal uremia. Experiments from this laboratory, presented in a preliminary report in 1944 (2), and later detailed (3), showed that severe hemorrhagic or traumatic shock in dogs could cause almost complete cessation of renal blood flow. Lauson, Bradley and Cournand (4) at about the same time reported decreased renal blood flow in human cases of shock. Since these effects could occur while the arterial blood pressure was as high as 80 to 100 mm. it appeared that the decrease in renal blood flow was due to vascular constriction (2, 3). Of the different hypotheses that had been advanced concerning the cause of the renal damage produced by shock (see Lucké, 5), the demonstration of decreased renal blood flow supported the hypothesis that renal ischemia existing during the shock state is a cause of subsequently persisting renal failure (2, 3).

To test this hypothesis further, renal ischemia was produced in dogs (2) by ligating the renal arteries for varying periods, and the after effects on renal function were observed. Ischemia of two-hours' duration was followed by temporary depression of urea clearance to nearly zero, but gradual recovery followed during a period of two or three weeks, and renal function might be completely restored. Ischemia of over four-hours' duration uniformly caused irreversible renal injury leading to death in uremia in four to eight days. Histological examinations by Dr. Jean Oliver showed that the glomeruli appeared to be normal, but that, in the distal tubules, there were changes resembling those seen in cases of uremia following the crush syndrome.

The present paper presents details of, and additions to, the original experiments performed to determine the duration of renal ischemia necessary to cause irreversible damage. As will be seen, dogs survived renal ischemia for two hours.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research. The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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Depending on environmental temperature, nutritional state of the animal, etc., three hours of renal ischemia might or might not be fatal.

At the time these experiments were undertaken, except for one paper by McEnery, Meyer and Ivy (6), no data could be found in the literature to indicate the duration of renal ischemia required to cause irreversible injury of the kidneys in dogs. McEnery *et al.* state, "Dogs may or may not survive a 30- to 60-minute period of ischemia of both kidneys". Scarff and Keele (7), confirmed recently by Badenoch and Darmady (8) found that one or two hours of occlusion of the renal artery produced irreversible renal damage in rabbits. To judge by comparison with our results, irreversible damage to the kidneys is caused by ischemia of somewhat shorter duration in rabbits than in dogs.

METHODS

1. *Method of producing renal ischemia.* The following procedure was applied with aseptic precautions to a series of female dogs under sodium pentobarbital anesthesia.

Through a midline abdominal incision the right kidney was excised. The left kidney was mobilized by dividing between silk ligatures the parietal peritoneum and the accompanying capsular vessels about one inch from the kidney. The renal artery was dissected free down to its origin from the aorta for a distance of $1\frac{1}{2}$ to 2 cm. Two serrefines, with jaws covered by rubber tubing and with springs of sufficient strength to obliterate the arterial lumen without damaging the intima, were then applied to the renal artery. The time of application of the serrefines was noted and the wound closed in layers with silk. In none of our experiments was the renal vein or the ureter obstructed. The presence of the ureteral vein and artery was noted at operation but it was felt unwise to ligate the ureteral artery even though in some instances it was obvious that some blood was still entering the kidney through this small vessel.

Following the removal of the serrefines the kidney was tacked in its original position by mattress sutures placed through the renal peritoneum and the dorsal abdominal wall at the four poles of the kidney. The abdominal incision was closed with layered silk sutures.

2. *Chemical determinations.* The blood urea nitrogen concentration was followed in all experiments by the manometric hypobromite method of Van Slyke and Kugel (9). In experiments performed in the months of June and July 1944 continuous 24-hour urea clearances were also performed. Urine urea determinations were made by the method of Van Slyke and Cullen (10). In this series of experiments urea clearances were performed before the serrefines were applied to the renal artery and for the two hours after release of the serrefines, at which time the animal was still under sodium pentobarbital anesthesia. Subsequently the animals were placed in metabolic cages and 24-hour collections of urine obtained. The urea clearances were followed until the death of the animal or until the clearance had returned to the normal range.

RESULTS

Experiments Performed in January, February and April 1943

1. *Renal ischemia for one hour.* Six dogs subjected to renal ischemia for one hour survived more than 30 days.

2. *Renal ischemia for two and three hours.* Six dogs were subjected to renal ischemia for two hours and six dogs were subjected to renal ischemia for three hours. One animal in this series died on the fourth post-operative day with a large *B Coli* abdominal abscess, the remainder of the dogs in this series survived more than 30 days.

3. *Renal ischemia for four hours.* Of six dogs subjected to renal ischemia for four hours, three lived for more than 30 days; three dogs died after 4, 4 and 10 days with blood urea nitrogen concentrations at death of 157, 182 and 320 mgm. per 100 cc., respectively.

4. *Renal ischemia for six hours.* Two dogs were subjected to renal ischemia for six hours. Both died in uremia after 4 and 10 days, respectively.

5. *Bilateral nephrectomy.* Two dogs were subjected to removal of both kidneys. They died on the fourth and eighth day, respectively, in uremia, verifying the observations of Hoff, Smith and Winkler (11).

Experiments Performed in June and July 1944

1. *Renal ischemia for two hours.* Five dogs were subjected to renal ischemia for two hours; all survived for over 30 days.

2. *Renal ischemia for three hours.* Of five dogs subjected to renal ischemia for three hours, two dogs survived for over 30 days; the three dogs that died survived 6, 9 and 15 days and the blood urea nitrogen concentrations at death were 374, 361 and 462 mgm. per 100 cc., respectively.

In our first series of experiments with renal ischemia of three hours' duration it will be noted that the experiments were performed in the winter and spring of the year when the environmental temperature was never above 80° F., while the second series of experiments were performed in the summer months when the environmental temperature was often above 95° F. and the humidity was also greatly increased. It appears that the increased environmental temperature and humidity in the summer months may have been responsible for the increased mortality of the dogs subjected to three hours of renal ischemia during this time of the year (cf. Lusk, 12, p. 151-155).

Blood Urea Changes

In figure 1 the plasma urea nitrogen concentration changes in one animal which had a bilateral nephrectomy are compared with the plasma urea nitrogen changes in three dogs subjected to renal ischemia of three hours' duration. The nephrectomized dog, K-7, died on the fifth day. K-91 and K-90, subjected to three hours of renal ischemia, died on the sixth and fourteenth day, respectively. These experiments were performed in the summer months. K-11, subjected to

three hours of renal ischemia in April, survived for over 30 days. The urea concentration curve of *K-11* is quite typical of the type of curve seen in all animals that survive. It will be noted in this animal that the blood urea rose at a steady rate for three days then remained practically stationary for two days and subsequently decreased. Histological studies of the kidneys of dogs subjected to renal ischemia for one, two and three hours will be reported elsewhere by Dr. Jean Oliver. However, we can state that the histological evidence for regeneration of

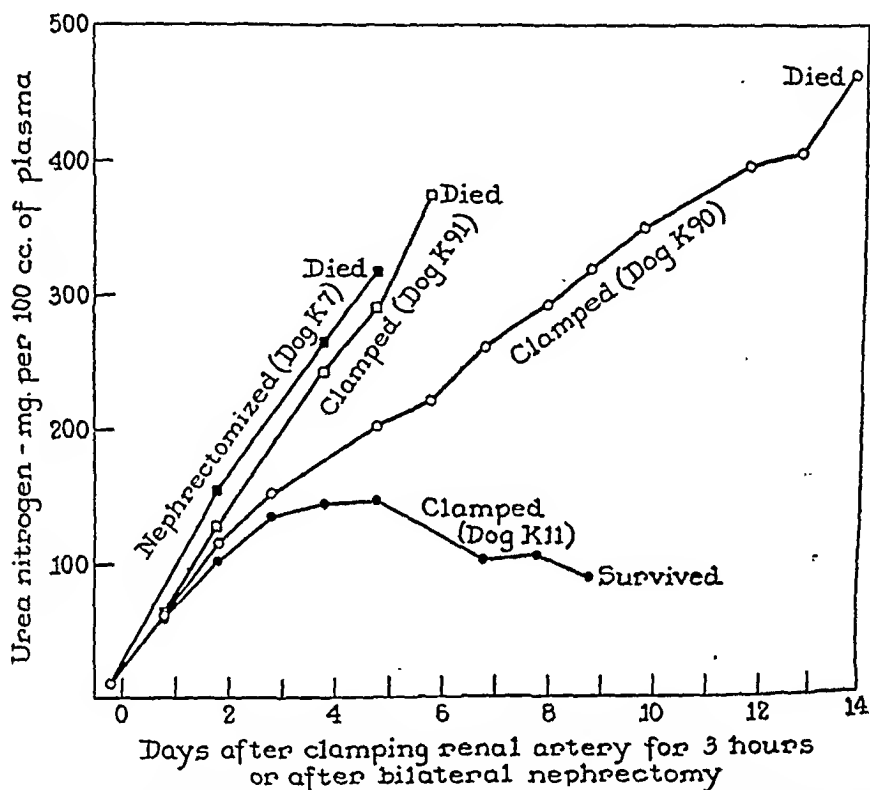


FIG. 1. COURSE OF CHANGES IN PLASMA UREA NITROGEN CONCENTRATION in a bilaterally nephrectomized Dog (K 7) compared with changes in three dogs subjected to renal ischemia of three hours' duration.

kidney tissue subjected to renal ischemia does not appear before the third day after induction of ischemia.

Urea Clearance Studies Following Renal Ischemia

Figure 2 compares the urea clearance and plasma urea nitrogen concentration in *dog K-89* subjected to two hours of renal ischemia on June 28, 1944. There is a steady improvement of the urea clearance in the first three days following induction of ischemia, although during this time there is a steady increase in the blood urea nitrogen. Although renal function was improving from the day of operation, three days were required before the improvement progressed far enough to stop the rise of blood urea. The urea clearance rose in about 35 days after induction of ischemia, to a level that was estimated, from the experiments

of Rhoads, Alving, Hiller and Van Slyke (13) to be normal for a one-kidney dog of its size.

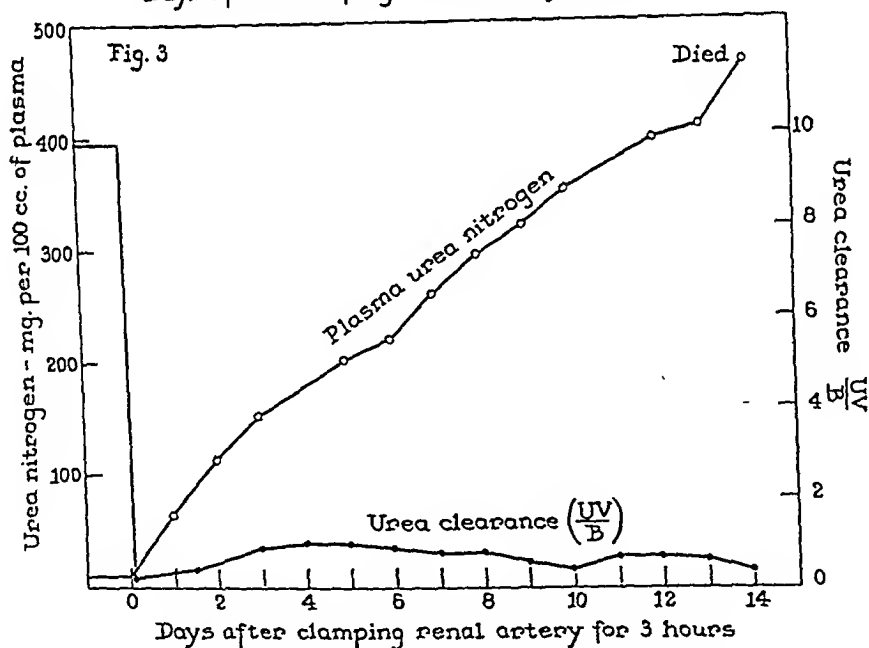
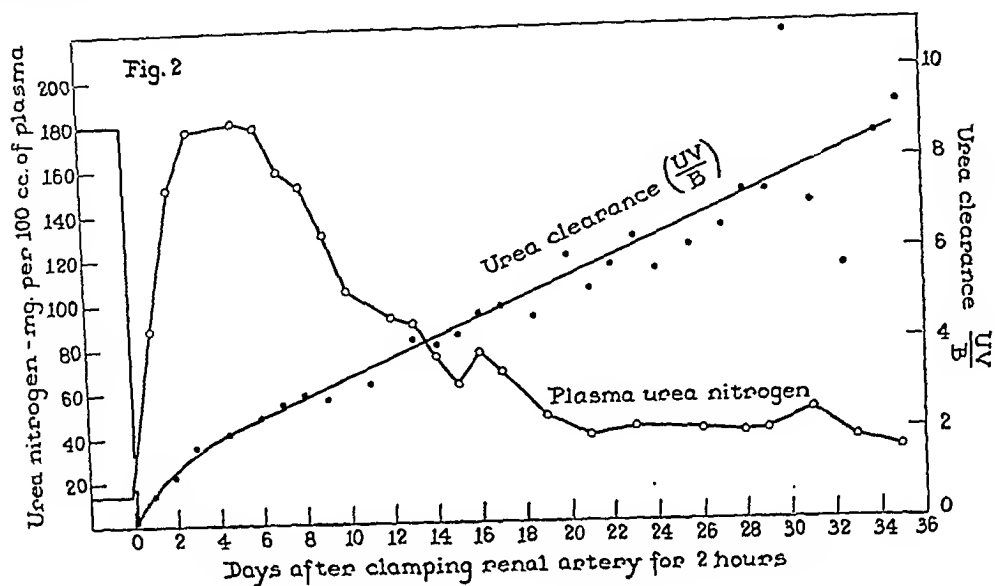


FIG. 2. COURSE OF CHANGES IN UREA CLEARANCE and plasma urea nitrogen concentration in Dog K 89 after two hours of renal ischemia.

FIG. 3. COURSE OF CHANGES IN UREA CLEARANCE and plasma urea nitrogen concentration in Dog K 90 after three hours of renal ischemia.

Figure 3, K-90, compares urea clearance with plasma urea nitrogen concentration in a dog which survived for 14 days after being subjected to three hours of renal ischemia. In contrast to K-89, this animal after the first three days following

three hours of renal ischemia showed no tendency for improvement of the urea clearance, after the fifth day there was a slow downward trend.

SUMMARY

1. Dogs with the right kidney removed uniformly survived clamping of the left renal artery for two hours. Some dogs survived clamping of the renal artery for three or four hours. Death in uremia regularly ensued with longer clamping of the renal artery.

2. In dogs subjected to three hours of renal ischemia the mortality was greater during summer, with high environmental temperature and humidity, than during winter, with low temperature and humidity.

3. After the clamp was removed from the renal artery, the urea clearance was extremely low, of the order of one to 10 per cent of normal, and blood urea began to rise rapidly. If recovery ensued, a steady rise of renal function occurred as measured by the urea clearance, and the clearance eventually reached a normal level, although a month was sometimes required for it to do so. If recovery did not take place, renal function remained at a low level, with progressive rise in blood urea nitrogen terminating in uremia and death.

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EFFECT OF 20, 60 AND 120 MINUTES OF RENAL ISCHEMIA ON GLOMERULAR AND TUBULAR FUNCTION¹

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Received for publication February 24, 1948

In other papers (1, 2) it has been demonstrated that renal ischemia of two-hour duration, caused by clamping the renal arteries of dogs, although not quite sufficient to cause irreversible injury, was followed by a period during which the urea clearance was depressed to a small fraction of its normal value, the blood urea nitrogen rose, and normal function was regained only in the course of two or three weeks.

The fall in urea clearance might be due to 1) decrease in the renal blood flow, or 2) decrease in the proportion of plasma water, with its urea and other crystalloid solutes, filtered in the glomeruli or 3) increase in reabsorption of urea in the tubules, such as might occur if the tubular walls were so devitalized by the ischemia that they could not prevent back diffusion of urea with reabsorbed water.

In an attempt to determine which of the three factors were responsible, experiments of the following type were performed. The right kidneys of female dogs were removed, and the dogs were infused with a solution of para-amino hippuric acid (hereafter designated as PAH) and creatinine, as described in a previous paper (3). The left kidneys were subjected to ischemia by clamping the renal arteries for periods of 20, 60 or 120 minutes. At intervals up to two hours after removal of the clamps blood was drawn simultaneously from the renal artery and the renal vein, analyses of the plasma from these bloods were made for creatinine and PAH and the rates of excretion of both substances in the urine were determined.

Of the three factors considered as possible causes for the persisting low urea clearance after renal ischemia, the results of the experiments could be expected to give indications as follows.

1. Renal blood flow could be calculated from the PAH values by the Fick principle, as $(\text{PAH excreted per minute})/(\text{PAH removed by the kidneys from one cc. of renal blood})$. This principle of measuring renal blood flow was introduced by Van Slyke, Rhoads, Hiller and Alving (4), who used urea as the measured

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research. The results were in part presented in 1944 by the authors and collaborators (1) in a report on the effects of shock on the kidney. The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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excretory substance. However, since only about 12 per cent of the blood urea is extracted by the kidneys, while an average of 87 per cent of plasma PAH is extracted (1, 3, 5) the PAH extraction can be measured more accurately. Previous experiments (1, 3) have shown that consistent values for renal plasma flow are obtained by the Fick principle, with PAH as excretory substance.

2. The fraction of plasma water filtered could be estimated from the fraction of creatinine extracted from the renal plasma, *provided* that the ischemia does not so injure the tubules that they permit some of the creatinine filtered in the glomerulus to diffuse back into the blood with the water reabsorbed from the tubular lumina. Previous studies (6) have indicated that creatinine in the normal dog is excreted entirely by glomerular filtration, without significant tubular reabsorption.

3. The functional state of the tubular cells could be indicated by the completeness with which the kidneys remove PAH from the renal blood plasma. Since normally an average of 87 per cent is removed (1, 3) while only 20 to 30 per cent can be considered to be filtered in the glomerulus (6), it follows that the greater part of the PAH removed from the plasma is normally excreted by the tubules. If, after renal ischemia, the tubules can at once resume extraction of PAH so complete that 87 per cent is removed from the renal plasma, it can be assumed as probable that the tubular cells are undamaged. If, however, there is a severe decrease in the PAH extraction, it may be taken as evidence of deranged tubular function and would suggest the possibility that the renal malfunction may include increased permeability to back diffusion of urea from the tubular lumina to the blood. Normally the tubules limit back diffusion of urea to about 40 per cent of that filtered (6, 7).

METHODS

1. *Operative and experimental procedures.* Female dogs were used. After anesthetizing with sodium pentobarbital, the spleen and right kidney were excised through a midline abdominal incision. The peritoneum and adventitia overlying the left renal vein were then dissected free from the vein so that samples of blood could be readily obtained. The parietal reflection of renal peritoneum was then excised between the upper and lower poles of the left kidney to permit exposure and dissection of the artery for a distance of one half to two cm., sufficient to accommodate the serrefines applied subsequently for the production of renal ischemia. A urethral catheter was inserted into the bladder and strapped in place. A ureteral catheter was inserted into the fore-limb vein and was connected to a continuous intravenous infusion pump, for the infusion of the solution of creatinine and PAH (3).

The technique for obtaining blood and urine samples was as described by Phillips *et al.* (3). Blood was drawn from the renal vein with a syringe provided with a curved No. 20 hypodermic needle; the needle was bent in a 90° curve so that its point was at right angles to the syringe axis. This facilitated puncturing the renal vein to obtain the sample. Care was taken to avoid undue tension on the kidney, its vessels and the ureter at the time of sampling, and in addition

care was taken to avoid any tension on the abdominal vena cava at the time of sampling.

In these experiments it was of interest to follow the clearance and extraction of creatinine and PAH immediately after release of the serrefines applied to the renal artery. Consequently the peritoneum and capsular vessels at the upper and lower pole of the kidney were left undivided, to insure that the kidney would remain in its original position and that kinking of the ureter, renal vein and renal artery would be minimized. As pointed out in the preceding paper (2), some blood does enter the kidney by way of the capsular vessels (not divided in these experiments) and in some animals by way of the ureteral artery. However, the amount of such vascular supply is small compared to the supply obtained through the renal artery.

Priming, followed by sustaining, infusions of creatinine and PAH were given, and one hour was allowed for attainment of equilibrium of creatinine and PAH between plasma and cells (3). One hour after start of the sustaining infusion three urine samples, collected during successive periods of 10 to 15 minutes each, were obtained. Blood was drawn from the femoral artery before the start of the first urine period and at the end of the third urine period. A sample of renal venous blood was obtained in the middle of the second urine period. After these control observations serrefines with rubber-covered jaws were applied to the renal artery for varying periods of time, which, in 3 series of experiments were approximately 20 minutes, 60 minutes and 120 minutes respectively. Ten minutes after release of the serrefine a sample of arterial blood was drawn and urine samples were obtained every 15 minutes for the subsequent two or more hours. Arterial blood samples were obtained at frequent intervals from the femoral artery. Renal venous blood samples were obtained at approximately 20, 60 and 120 minutes after release of the serrefines.

During application of the serrefines the sustaining infusion was discontinued. When renal ischemia was maintained for 120 minutes bladder washings for 110 minutes of this time were obtained. The smallness of the amounts of PAH found in these bladder washings, obtained during the period of ischemia, was a further check on the completeness of the renal ischemia. In two experiments both kidneys of each dog were excised and the bladder clearance of PAH (clearance estimated from PAH in bladder washings of the nephrectomized dogs) was determined with plasma PAH concentrations of 4-6 mgm. per 100 cc. The bladder clearance was found to be in the range of 0.02-0.04 cc. per minute. In the experiments here reported the PAH clearance during 120 minutes of renal ischemia were never more than 10 times the bladder clearance, indicating that the renal ischemia was nearly complete.

2. *Method of administration and analysis of creatinine and PAH.* The techniques used for administering creatinine and PAH and for analyzing plasma, blood and urine for these substances have been described previously (3). PAH concentrations found in plasma of renal venous blood were corrected as previously described (3), for diffusion of 5 per cent of the plasma PAH *in vitro* from plasma to erythrocytes during the drawing and centrifuging of the blood.

RESULTS AND DISCUSSION

Renal blood flow. The results in table 1 indicate that, after renal ischemia for periods up to two hours, when the clamps were removed from the renal arteries blood flow through the kidneys was soon reestablished. Renal plasma flows, measured 90 to 120 minutes after release of the clamps, averaged 81 to 85 per cent

TABLE 1. *Effects of clamping renal artery for varying lengths of time on subsequent renal function (one-kidney dogs)*

DURATION OF CLAMPING	DOG NO.	% OF EXCRETORY SUBSTANCE EXTRACTED FROM RENAL PLASMA						RENAL PLASMA FLOW ¹			RENAL PLASMA FLOW ESTIMATED AS 1.15 x PAH CLEARANCE		
		Para-amino hippurate			Creatinine			Before clamping	15-20 min. after release of clamp	90-120 min. after release of clamp	Before clamping	15-20 min. after release of clamp	90-120 min. after release of clamp
		Before clamping renal artery	15-20 min. after release of clamp	90-120 min. after release of clamp	Before clamping	15-20 min. after release of clamp	90-120 min. after release of clamp						
min.								cc/min.	cc/min.	cc/min.	cc/min.	cc/min.	cc/min.
20	N2	0.91	0.85	0.88	—	—	—	157	96	48	164	94	49
20	N3	0.85	0.79	0.73	0.33	0.21	0.21	48	85	85	47	97	71
20	N4	0.80	0.57	0.61	0.30	0.23	0.38	33	22	18	30	14	13
20	N9	0.90	0.90	0.72	0.17	0.22	0.26	61	58	46	63	60	38
Mean % of value before clamping		100	90	85	100	90	118	100	101	85	100	91	71
60	N6	0.76	0.39	0.50	0.33	0.15	0.20	67	37	66	59	17	38
60	N7	0.83	0.67	0.71	0.22	0.16	0.25	99	98	76	94	29	46
60	N8	0.81	0.31	0.38	0.22	0.05	0.15	96	67	72	90	24	31
Mean % of value before clamping		100	57	66	100	47	72	100	75	84	100	29	49
120	K69	0.90	0.11	0.11	0.23	0.02	0.02	87	57	76	90	7	9
120	K67	0.91	—	0.20	0.19	—	0.05	93	—	56	96	—	13
120	K68	0.94	—	0.43	0.22	—	0.14	42	—	36	46	—	17
120	K70	0.92	0.16	0.13	—	—	—	116	52	90	122	9	14
Mean % of value before clamping		100	—	24	100	—	37	100	—	81	100	—	18

¹ Renal plasma flow measured as (mgm. PAH excreted per min.) / (mgm. PAH extracted from 1 cc. of plasma).

of the pre-ischemic flows, whether the period of ischemia had been 20, 60 or 120 minutes. The decrease of urea clearance nearly to zero, noted previously (1) as a sequence of clamping the renal artery for two hours, was evidently not due to failure of blood flow to return to the kidneys after the clamps were removed.

PAH extraction and tubular damage. The PAH extraction values indicate that tubular function was severely deranged by two hours of ischemia. The damage was progressive with the duration of the ischemia, as indicated by the progressive

decrease in the fraction of PAH extracted from the renal plasma; this fraction averaged 85 per cent of its pre-ischemic value after 20 minutes ischemia, 66 per cent after 60 minutes and 24 per cent after 120 minutes of ischemia, the extractions being averages measured 90–120 minutes after release of the arterial clamps. This functional evidence of tubular damage agrees with the results of histological examination of the kidneys by Dr. Jean Oliver, mentioned in a previous report (1).

Tubular reabsorption as a cause of renal failure after renal ischemia and shock. The functional effects of tubular injury have been studied by Richards (8), who observed *in vivo* the activities of the nephrons of frogs that had been poisoned by mercury and other nephrotoxic substances. He found that in the glomeruli blood flow and filtration went on at a fully normal rate, but that the filtrate was completely absorbed from the tubular lumina, so that no urine reached the bladder. Anuria resulted, not from failure of filtration, but from complete reabsorption of the filtrate. The poisoned tubular walls permitted unselective diffusion of the entire filtrate, presumably drawn by the osmotic attraction of the plasma proteins, back into the circulation. The tubular walls apparently had become devitalized, so that they acted like inert permeable membranes.

In view of the absence of evidence of marked glomerular or vascular injury and of the positive evidence, functional and histological, of tubular injury, it appears probable that tubular reabsorption of the type observed by Richards (8) causes the urea retention that follows sufficiently prolonged periods of renal ischemia, as exemplified by figure 1 (reversible) and figure 2 (irreversible) of the preceding paper (2).

Lucké (9) has noted in kidneys from cases of uremia following shock from war injuries that the type of tubular injury was identical with that observed in cases injured by nephrotoxic poisons, and points out, from analogy with Richards' observations, that post-shock uremia is probably the effect of tubular injury caused by ischemia suffered during the shock, the renal failure being due to unselective tubular reabsorption of the type observed by Richards. Badenoch and Darmady (10) have compared side by side sections from the kidneys of patients dying in post-shock uremia with sections from the kidneys of rabbits in which uremia was caused by two-hour ligation of the renal arteries, and have found the type of tubular lesions practically identical. Results in previous papers from this laboratory (3) have shown that hemorrhagic and traumatic shock of severe grade causes nearly complete cessation of blood flow through the kidneys of dogs.

These observations, taken with the present data showing that ischemia prolonged over periods of hours comparable to the duration of shock in severe untreated cases produces progressive damage to tubular function, indicate the probability that post-shock uremia is caused by reabsorption of excretory products through damaged tubular walls. It also appears, from Lucké's (9) observation of the identical nature of the tubular lesions in post-shock uremia and in poisoning by sulfonamides and other nephrotoxic agents, that ischemia is one of many nephrotoxic agents that produce a common picture of tubular damage with resultant renal failure from tubular reabsorption.

Cause of the decreased extraction of creatinine from the renal plasma after ischemia. In the kidneys of the normal dog excreting ordinarily large volumes of urine there is evidence that creatinine and inulin do not pass to a measurable extent in either direction through the tubular walls, so that the fraction of either removed from the renal plasma is the fraction that is filtered out in the glomeruli (6, 11). It has been rather generally assumed that these relations hold also in abnormal kidneys, the clearance of creatinine or inulin in dogs and inulin in man serving as a measure of glomerular filtration. On such an assumption, a great decrease in the extracted fraction of creatinine, such as is seen in table 1 after two-hour ischemia, without a commensurate decrease in blood pressure, would be taken as evidence of decreased filtration due to decreased blood pressure in the glomerular capillaries, the fall in pressure being attributable to dilatation of the efferent vessels from the glomeruli.

However, in view of the evidence of tubular damage in these animals, of Richards' (8) evidence that such damage can cause abnormal tubular reabsorption, and in view of the lack of evidence of efferent dilatation, it appears more probable that the decrease in the filtered fraction of creatinine is attributable to back diffusion of creatinine through the injured tubular walls into the circulation. The condition appears to be similar to that found by Richards, Westfall, and Bott (12) in a dog with kidneys previously damaged by uranium. Creatinine clearances were consistently lower than inulin clearances. The results were interpreted by Richards *et al.* as evidence of back diffusion of creatinine through the injured tubular walls, inulin back diffusion being less because of lesser diffusibility of the large inulin molecule.

Interpretation of clearances as measures of renal blood flow and glomerular filtrate in conditions of tubular injury. Renal blood flow. Smith and his colleagues have shown for normal men and dogs that the clearances of diodrast (13) and PAH (14) approach so nearly to otherwise estimated values of the renal plasma flow that it is evident that their extractions from the renal plasma must be nearly complete, and that their clearances, in terms of cc. of plasma cleared of diodrast or PAH per minute, may therefore be assumed to approximate the volume of plasma flowing through the kidneys. These conclusions have been confirmed for subjects with normal kidneys; in both dogs (1, 3) and men (5) simultaneous determinations of PAH in arterial and renal venous plasma have shown that an average of 87-88 per cent of the PAH is normally extracted by the kidneys. Hence, when the kidneys are normal, one can estimate the renal plasma flow as 1.15 times the plasma PAH clearance, usually with but a small per cent error (3). This relation was shown to hold in acute hemorrhagic and traumatic shock so severe that the renal blood flow was temporarily reduced to a small fraction of normal (3). However, the results in the present paper show that when renal ischemia is sufficiently complete and prolonged to cause severe tubular injury the relation no longer holds. Comparison of the last three columns of table 1 with the preceding three columns shows that 1.15 times the PAH clearance approximately equaled the measured renal plasma flow in observations made before the renal artery was clamped, and after release of the clamps when the

latter had been applied for only 20 minutes. But when the clamps had been applied for two hours the subsequent values of 1.15 times PAH clearance averaged less than one fourth of the measured renal plasma flow. It is evident that when the tubules are injured PAH extraction may be so diminished that the PAH clearance indicates only a small fraction of the renal blood flow. Presumably the same limitation applies also to diodrast clearances. Smith has been careful to state that PAH and diodrast plasma clearances will fall short of renal plasma flows in proportion as the fraction of PAH or diodrast extracted from the renal plasma falls short of unity. The present results indicate the need of keeping this reservation in mind, particularly in conditions where tubular injury may be present.

Glomerular filtrate. In normal dogs there is evidence that the tubular walls neither excrete nor reabsorb creatinine and inulin (6); hence the fraction of either extracted from the renal plasma by the kidneys may be taken to be the fraction filtered in the glomeruli. On the evidence of the classical experiments of Richards and his collaborators (15) showing that plasma crystalloids and water are filtered in the same proportions that they have in the plasma, the fraction of creatinine or inulin extracted can be taken as a measure also of the fraction of plasma water filtered, as long as the tubular walls retain their normal impermeability to the creatinine and inulin. When the tubules are damaged, however, so that they cannot extract PAH with normal completeness from the plasma, it appears possible that the tubular walls may become sufficiently permeable to creatinine and inulin to permit back diffusion of these substances from the tubular lumina into the circulation, in the manner observed by Richards (8), so that the amounts excreted are much less than those filtered in the glomeruli. For reasons discussed above, we are inclined to interpret the very low extractions of creatinine noted after two hours ischemia (table 1) to tubular back diffusion. Although the lack of an independent measure of glomerular filtration makes the conclusion less certain than those reached with regard to the interpretation of PAH clearances as renal blood flows in the same experiments, it appears that interpretation of clearances of creatinine, and of such similarly excreted substances as inulin, mannitol, and thiosulfate, in terms of glomerular filtration rates is open to doubt in conditions where tubular damage may make reabsorption of these substances a possibility.³

It is of interest in this connection that the impermeability of even normal tubular walls to creatinine, in the case of man, appears to be not absolute. Chesley (16) has found that when dehydration shrunk the urine flow of human subjects below 0.35 cc. per minute, reabsorption of all the urine solutes, including creatinine, occurred.

³ In a publication from this laboratory (3) on the effects of acute shock on renal function of dogs, the fraction of creatinine extracted from the renal plasma was used as a measure of the filtered fraction of plasma water. In this case the assumption was presumably justified, since simultaneous determinations of the extracted fraction of PAH showed that this fraction was normal, 0.87 ± 0.04 , indicating that the degree of shock used had not yet significantly injured the tubules.

SUMMARY

Dogs have been subjected to renal ischemia by clamping the renal arteries for periods varying from 20 minutes to two hours, and the effects on the renal function have been studied by observations of the renal plasma flow and of the completeness with which para-amino hippurate and creatinine were extracted from the renal plasma during two hours after removal of the clamps.

Renal blood flow was quickly resumed at a nearly pre-ischemic rate.

The proportions of para-amino hippurate and creatinine extracted from the plasma were not markedly affected after 20-minute ischemia, but after two-hour ischemia they were reduced, the creatinine extraction, in 3 experiments, to 63, 26, and 9 per cent respectively of pre-ischemic values, the PAH extraction, in 4 experiments, to 37, 14, 11, and 10 per cent, respectively.

From the functional and histological effects of the two-hour ischemia, it appears probable that the decreases in extracted fractions were due to tubular injury, which decreased the proportion of plasma PAH excreted by the tubules, and increased tubular reabsorption of creatinine from the glomerular filtrate. The evidence supports Luek 's view that post-shock uremia is the result of tubular reabsorption of excretory products.

With tubular injury such as that caused by ischemia, PAH clearance does not serve as a measure of renal blood flow; and it appears doubtful that in the presence of such injury clearances of creatinine and similarly diffusible substances can be interpreted as measures of glomerular filtration rate.

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TRAUMATIC SHOCK XVI: AMINO ACID METABOLISM IN HEMORRHAGIC SHOCK IN THE DOG¹

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Received for publication October 6, 1947

A previous communication in this series (1) dealt with the possible relationship between derangements of intermediary carbohydrate metabolism and the progressive deterioration in traumatic shock. In this communication we present analogous data on intermediary protein metabolism in this disorder.

The rise in total blood amino nitrogen in traumatic shock (1-4) has been attributed to impairment of the deaminating function of the liver (2, 3) and to increased production of amino acids because of accelerated tissue breakdown (5). Defective deamination was inferred from the finding of decreased urea synthesis by liver slices taken from shocked rats (6) and the lack of sufficient urea accumulation in the blood of such animals subjected to nephrectomy followed by amino acid administration (7). Defective liver function in shock has been demonstrated (8, 9). Our objective is to identify the nature of the defect in liver function which is specifically related to the fatal character of the shock syndrome. If defective deamination occurs in shock, it is essential to our purpose to determine whether it is causally related to the progressive deterioration in this condition. If the rise in blood amino nitrogen consequent to shock is referable to failure of deamination, the administration of additional amino acid during shock to an animal already laboring under a defective deaminating mechanism should result in abnormal persistence of the amino acid in the circulation. Furthermore, if these abnormalities are intimately related to irreversibility of shock, they should not be favorably influenced by any therapeutic procedure which fails to result in survival. This report is concerned with experiments designed to test the foregoing concepts. The data indicate that *a*) no correlation exists between the development of irreversibility to transfusion and the ability to clear the blood of injected amino acid and *b*) any delay in clearance of injected amino acid from the blood during shock does not persist after transfusion, whether therapeutically effective or not and regardless of the blood amino-acid nitrogen level.

METHOD

Hemorrhagic shock was produced in morphinized mongrel dogs (8-12 kgm., fasted for only 12-24 hours) by a method previously described (10). Solutions of single amino acids in doses of 100 or 200 mgm. per kilogram were injected

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

intravenously before hemorrhage, during the oligemic phase of shock and following transfusion. The amino acids studied were d,l-alanine, glycine, d,l-cysteine hydrochloride, l-lysine monohydrochloride, l-tyrosine, l-tryptophane, d,l-aspartic acid. A proprietary amino acid mixture (casein hydrolysate fortified with tryptophane-Stearn) was also given in doses of 200 mgm. and 2.0 grams/kgm. The volume of the injected solutions varied from 10 to 100 ml., depending upon the solubility of the amino acid. Solution of tyrosine, tryptophane and aspartic acid was effected with the aid of sodium carbonate.

Arterial blood specimens were taken immediately before each injection of amino acid and at 15-minute intervals thereafter for one hour. Blood total amino acid nitrogen² was determined by the method of Frame, Russell and Wilhelmi (11), blood lactate by the method of Barker and Summerson (12), pyruvate by the method of Bueding and Wortis (13), urea by the method of Karr (14) and nonprotein nitrogen by the method of Koch and McMeekim (15), blood alanine by the method of Alexander and Seligman (16) and blood glycine by a modification of the method of Alexander, Landwehr and Seligman (17). In a few experiments aspartic acid was determined by the method for alanine (16). By comparison with standards of aspartic acid, this method gave values which were approximate only, but of sufficient accuracy in the presence of large concentrations of aspartic acid to be worthwhile.

RESULTS

Data and results are summarized in table 1 and figures are presented of representative experiments. Detailed analysis of the results for each amino acid follows.

d,l-alanine tolerance tests (seven dogs in irreversible shock). (1) In the *normal state*, i.e., prior to the induction of shock, the injection of alanine in the two dosages used (table 1) increased total blood amino-acid nitrogen 3-5 and 5-10 mgm. per cent, respectively (figs. 1 and 2). In five of seven dogs the maximum increase was immediate and a steady decline occurred thereafter. In two animals the maximum rise did not occur until one hour after alanine injection. After one hour some 50-75 per cent of the increase had been dissipated in four dogs, 100 per cent in one. Alanine nitrogen paralleled the blood amino acid nitrogen except in one instance (fig. 1) in which the latter remained high while the alanine fell to normal. This plus the fact that urea did not increase suggests a transfer of the alanine amino nitrogen to form some other amino acid.

(2) During the *oligemic phase* of hemorrhagic shock, the injection of alanine in six dogs produced a somewhat greater immediate increase in blood amino nitrogen

² The whole blood amino acid nitrogen will correctly reflect shifts in the plasma amino acid nitrogen so long as the hematocrit remains essentially constant. In these shock experiments the hematocrit does not vary significantly. Hemodilution causing a drop in hematocrit from 45 to 35 per cent packed cells, which represents an extreme situation, would effect a drop in blood amino nitrogen from, e.g., 8.7 to 8.1 mgm. per cent, if the ratio (2) of amino acid in red blood cells to that in plasma is taken as 2 for the purpose of this calculation.

TABLE 1. PROTOCOL DATA AND SUMMARY OF RESULTS

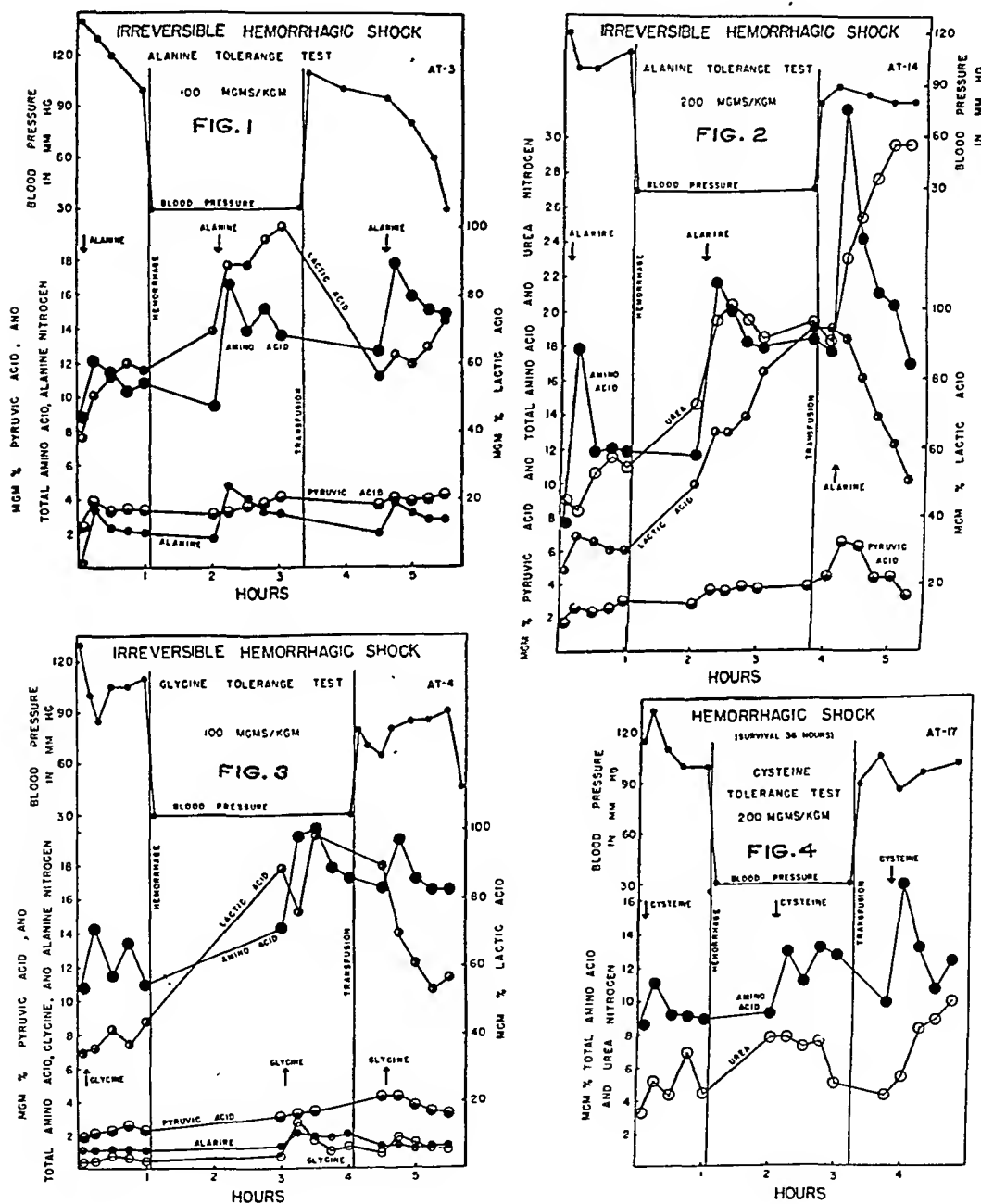
AMINO ACID	EXP. NO.	FIG. NO.	REVERSIBLE OR IRREVERSIBLE SHOCK	AMINO ACID DOSE	NORMAL STATE		HEMORRHAGIC SHOCK		AFTER TRANSFUSION	
					Amino acid clearance ¹	Increase in blood level of urea ²	Amino acid clearance ¹	Increase in blood level of urea ²	Amino acid clearance ¹	Increase in blood level of urea ²
d,l-Alanine	1		I	100	good				good	
	2		I	100	poor ³		fair		poor	
	3	1	I	100	fair		fair		fair	
	6		I	200	fair		good		fair	
	13		I	200	good	++	poor	0	fair	++
	14	2	I	200	fair	+	fair	++	good	++
	15		I	200	poor ³	0	good	++	good	++
Glycine	4	3	I	100	good		fair		good	
	5		I	100	good		fair		good	
d,l-Cysteine	16		I	100	good	++	good	++	good	++
	17	4	R?	200	good	++	poor	0	good	++
l-Lysine	22	5	R	200	good	0	fair	++	good	++
	26		I	200	good	0	good	++	good	++
l-Tyrosine	18		R	100	fair	++	poor	++	good	++
	19	6	I	200	good	0	poor	++	good	++
	20		I	200	fair		good		fair	
l-Trypto- phane	21	7	I	200	good	0	good	++	good	++
	24		I	200	good	++	fair	++	good	++
d,l-Aspartic	7		R	100	good		poor		good	
	8		I	100	good		poor		good	
	10	8	I	100	good		poor		fair	
	11		R	100	good		good		fair	
	12		I	100	good		poor		poor	
Amino Acid Mixture	27	9	I	200	fair	+	fair	0	good	+
	28		I	200	good	++	fair	+	good	++
	29		I	300	good	++	poor	++	fair	+
	30		I	2000	good	0				
	31		I	2000			fair	0		
	32		I	2000			fair	0	fair	0
TOTAL.....					19 good	6 0	7 good	5 0	17 good	1 0
					6 fair	2 +	11 fair	1 +	8 fair	2 +
					2 poor	7 ++	7 poor	10 ++	2 poor	12 ++
TOTAL IMPRESSION.....					good	+	fair	+ to ++	good	++

¹ Amino acid clearance as indicated by determination of the total amino acid nitrogen. If the blood amino nitrogen levels one hour after injection were above the starting level by an amount equal to: 0-25% of the total rise, the clearance was considered 'good'; 25-75% of the total rise, the clearance was considered 'fair'; 75-100% of the total rise, the clearance was considered 'poor.'

² Urea synthesis was suggested by sharp elevations in blood levels following amino acid injection. In normals the rises in urea were not as high as in shock due to good renal function. Increases in the level of urea nitrogen from the time of amino acid injection are defined as 0 for 0-1 mgm.% rise; + for 1-3 mgms.% rise; ++ for above 3 mgms.% rise.

³ In these experiments alanine was cleared normally as indicated by alanine determinations. Alanine nitrogen must have been transferred so as to form another amino acid.

than in the normal state (figs. 1 and 2). This was also true of the blood alanine nitrogen level in the three dogs in which alanine was determined. After one hour, from 50-100 per cent of the increase in blood amino acid nitrogen was still present



FIGS. 1-4

in four experiments, and none of the increase was present in two. Fifty per cent of the increase in alanine was still present after one hour in all three animals in which alanine was determined.

Blood urea was studied in three dogs. There was a considerable increase in urea in two dogs (fig. 2). No urea synthesis occurred in the third, in which the increase in the amino acid level persisted during the period of observation.

(3) *After therapeutically ineffective transfusion.* All the foregoing seven dogs proved to be in irreversible shock. Alanine was injected one half to one hour after transfusion. In four dogs the amino acid was administered while the blood pressure was still above 80 mm. Hg; in three dogs the blood pressure was falling rapidly to 35 mm. The increase and decline of the amino acid nitrogen level after alanine administration was practically no different from that in the normal state in two dogs. Clearance was more efficient than in the normal in two others, and slower than in the normal in three, two of which were at a very low hypotensive level and died before the curve could be completed. Of the three dogs in which alanine was determined, two showed the same rate of decline in alanine nitrogen as in the normal state and in one (early death) the decline was slower than in the normal or oligemic phase.

Of three dogs in which urea curves were obtained, two showed some rise in blood urea; in the third animal urea nitrogen increased about 12 mgm. per cent (fig. 2).

(4) *Lactic and pyruvic acids.* Alanine injections caused a moderate rise (10 mgm. per cent) in lactate and a slight rise in pyruvate in the normal state and in two dogs a marked rise in lactic acid and in the lactate/pyruvate (L/P) ratio in the oligemic phase of hemorrhagic shock. The marked characteristic drop in lactic acid and in the L/P ratio following transfusion was followed in three dogs by a secondary rise in lactic acid following injection of alanine (fig. 1).

COMMENT. Alanine was cleared from the blood of the dog in shock somewhat more slowly than in the normal state in some dogs, but as well or even better than in the control state in others, especially after transfusion. The blood amino acid nitrogen level, usually elevated in shock, was only temporarily affected by the alanine. Urea, lactic acid and pyruvic acids were produced following the injection of alanine in the normal state, in the oligemic phase of hemorrhagic shock and following ineffective transfusion.

Glycine tolerance tests (two dogs in irreversible shock). In the normal state, both dogs exhibited complete disappearance curves of the increased amino nitrogen following glycine injection. In the first there was very little increase in glycine, but there was evidence of pyruvic and lactic acid formation (fig. 3). In the oligemic phase of shock glycine disappeared at the normal rate, but the increase in total amino acid nitrogen level consequent to the administration of glycine diminished at a slower than normal rate. Some alanine was produced (fig. 3). In the posttransfusion phase of irreversible shock the glycine disappearance curve was normal and the lactic acid and L/P ratio dropped precipitously as usual, but the total amino acid nitrogen level did not return to normal.

COMMENT. Despite the fact that injected glycine disappeared from the blood in shock at a normal rate, the elevation in total amino acid nitrogen progressed with the development of shock and continued high after transfusion.

d,l-cysteine tolerance tests (two dogs, one in irreversible shock). One dog was

irreversible to transfusion, the other (fig. 4) died in 36 hours (borderline reversibility). In the *normal state* the total amino acid level rose slightly following injection of the amino acid, and as it returned to normal in one hour, blood urea increased. In the *oligemic phase* of shock cysteine clearance was good in one animal and fair in the second (fig. 4). In this dog blood urea remained stationary. After *transfusion* the increase in total amino acid nitrogen level which followed the cysteine injection fell as well as in the normal state. In both dogs urea rose to high levels after amino acid injection, simulating the pattern seen in the normal state.

COMMENT. During the oligemic phase of shock, elevation in total amino acid nitrogen due to the injection of cysteine was transient, except in one dog. In this instance the failure of urea synthesis and amino acid clearance may be attributed to poor circulation through the liver, since both functions were normal after transfusion. The irreversibly shocked dog showed as good clearance as the dog in reversible shock. The usual rise in total amino acid nitrogen as shock progressed was perhaps less striking in dogs which received cysteine.

l-lysine tolerance tests (two dogs, one reversible and the other irreversible to transfusion). In the *normal state* the injection of lysine was followed by a rise in total amino acid nitrogen in only one animal. In this dog the preinjection level was regained in one hour. Urea did not accumulate significantly, but the non-protein nitrogen rose seven mgm. per cent. This suggests that lysine was converted to nitrogen-containing compounds which are free of α -amino carboxyl groups and not precipitated with deproteinizing agents (other than urea). In the second dog, no rise in total amino acid nitrogen occurred, but urea was formed. High urea levels were not reached since normal kidney function was still present (fig. 5).

In the *oligemic phase* of shock the injection of lysine was followed by normal amino nitrogen clearance in the first dog and a near normal one in the second. Urea rose in both and nonprotein nitrogen rose in the first.

Following *transfusion* the usually high basal amino acid nitrogen level was not present and the total amino acid nitrogen disappearance curve was normal. Urea production as seen in figure 5 occurred in both dogs. The nonprotein nitrogen rose 20 mgm. per cent above the normal level in the first dog.

COMMENT. Normal disappearance curves were seen in all phases of shock following injection of lysine in two dogs. Tolerance to injected lysine was the same in irreversible as in reversible shock.

l-tyrosine tolerance tests (three dogs, one reversible and two irreversible to transfusion). In the *normal state* the injection of tyrosine was followed by an immediate rise in total amino acid nitrogen in two dogs. In one of these, return nearly to the preinjection level occurred within one hour (fig. 6). Blood urea remained relatively unchanged. In the other animal the amino acid level was only slightly reduced after one hour. Lactic and pyruvic acid rose 24 and 2 mgm. per cent, respectively. In the third dog, no immediate rise in amino acid followed injection of tyrosine, but a small rise in urea was seen. The nonprotein nitrogen of two dogs did not rise significantly above starting levels.

In the *oligemic phase* of shock amino acid nitrogen rose further following the

injection of tyrosine, somewhat belatedly in one animal, steadily from the beginning in the second (fig. 6). In the third, return to preinjection level occurred. Rises in blood urea occurred in the first and second animals. The nonprotein nitrogen rose in the second and third.

Following *transfusion* a normal amino acid tolerance curve was observed in two animals (one in reversible shock and one in irreversible shock), but in the third the curve was only fair, as was the case in this dog in the normal state. The rise in urea was striking in the first and second dogs and the final amino acid level was not significantly elevated.

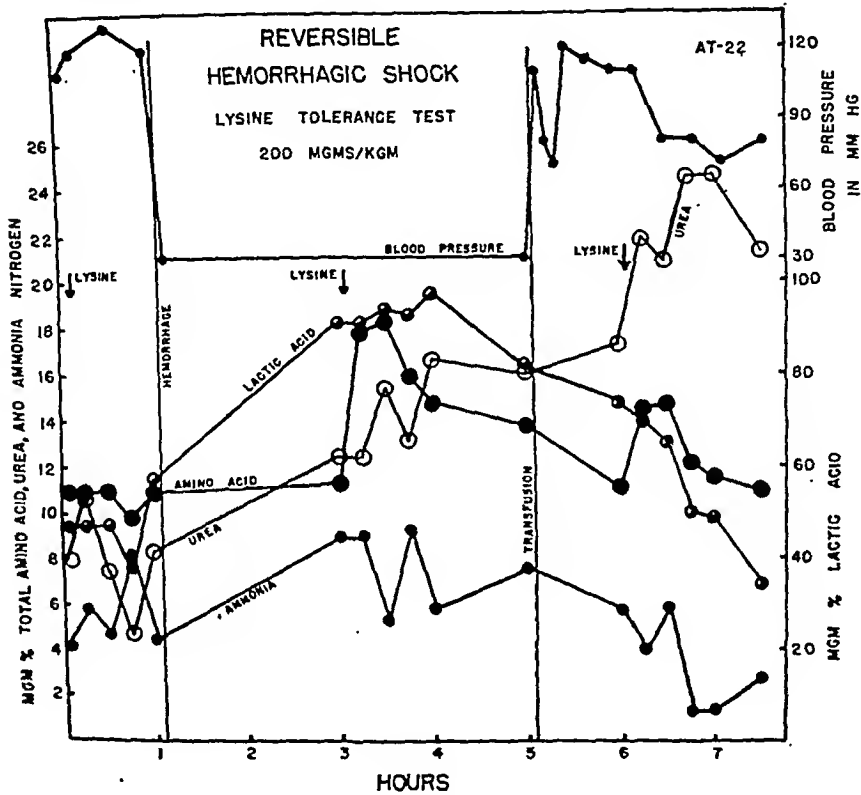


FIG. 5

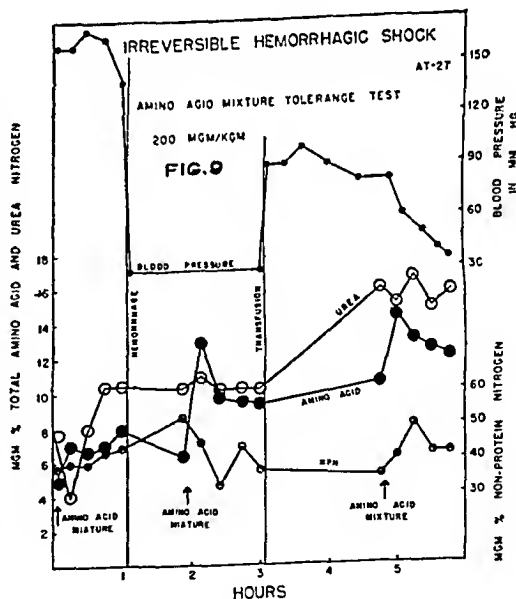
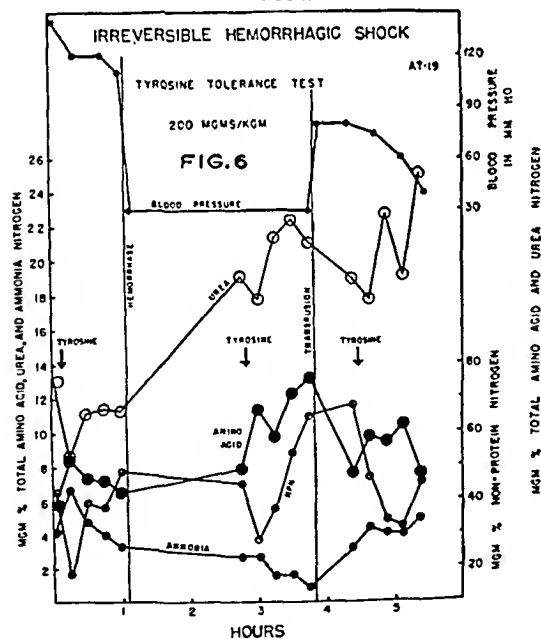
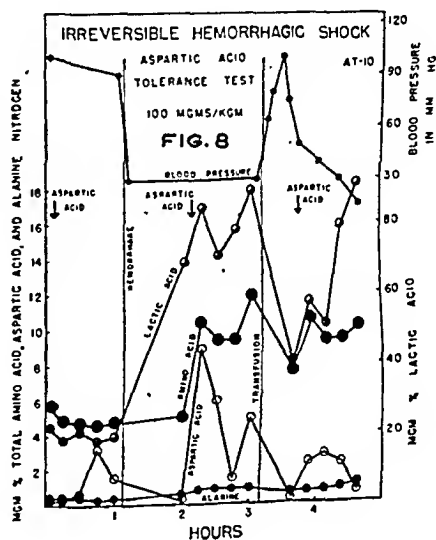
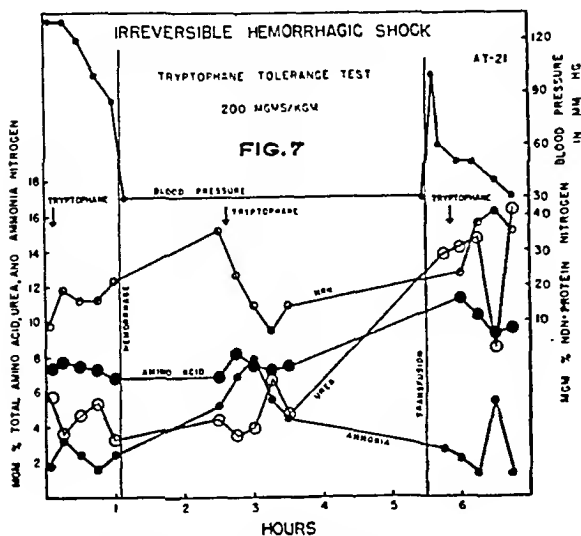
COMMENT. Variation in the tolerance curves following injection of tyrosine was noted in three normal dogs. In shock, two of three dogs showed poor blood clearance of amino nitrogen accumulation consequent to tyrosine administration. After transfusion, the clearance was good in all animals irrespective of whether the animals survived or died of shock. No correlation with irreversibility was demonstrable. The usual elevation in blood amino nitrogen associated with shock was not noted in these animals.

l-tryptophane tolerance test (two dogs in irreversible shock). In the normal state blood amino acid nitrogen was practically unchanged following the injection of tryptophane, but the nonprotein nitrogen rose, and in one dog urea concentration rose.

In the oligemic phase of shock the amino acid nitrogen rose slightly following

the injection of tryptophane and the preinjection levels were reached within an hour. Urea rose after amino acid injection (fig. 7).

After *transfusion* there was good clearance of amino acid nitrogen following rises produced by the injected amino acid. Rises in blood urea were also ob-



FIGS. 6-9

served following injection. In both dogs the blood pressure dropped precipitously to about 20 mm. Hg during the last tolerance test. In one the usual rapid drop in blood lactic acid did not occur.

COMMENT. Normal amino nitrogen disappearance curves following injection of tryptophane were observed in the normal state and in shock before and after transfusion.

d, l-aspartic acid tolerance test (five dogs, two reversible and three irreversible to

transfusion). In the *normal state* the injection of aspartic acid did not affect the amino acid nitrogen level materially. In two instances (fig. 8) a drop, and in three a small rise followed by a drop to the preinjection level, occurred within 45 minutes. In one instance a small rise in lactic acid occurred.

In the *oligemic phase* of shock total amino acid nitrogen rose following the administration of aspartic acid, and the change persisted in three animals (irreversible shock). In the fourth (reversible) a slight drop occurred following the rise, and in the fifth (reversible) the aspartic acid was cleared so rapidly that no rise above the usual shock level occurred. Clearance of the aspartic acid as such, determined approximately by a modification of the alanine method (16), occurred during irreversible (two of three instances) and reversible shock (one of two instances). Lactic acid production was observed in two dogs and slight rises in alanine were also noted in one dog.

Following *transfusion* amino acid nitrogen returned to a normal level in one of two dogs in reversible shock in spite of the injection of aspartic acid, but in the three irreversible dogs in which rapid circulatory failure occurred, the amino acid level remained elevated. Good clearance occurred in one of the irreversible dogs, but in the other two in which the clearance was poor and fair in terms of total amino acid nitrogen, clearance of aspartic acid was demonstrated by aspartic acid determinations. Lactic acid production occurred in three dogs following injection of aspartic acid (fig. 8).

COMMENT. Rises in blood amino acid nitrogen resulting from injected aspartic acid persisted during the oligemic phase of hemorrhagic shock (except in one dog that recovered following transfusion). Better clearance of aspartic acid was shown by aspartic acid determination than by total amino nitrogen measurement. This suggests conversion of aspartic acid to another amino acid or fixation of its free carboxyl group so that decarboxylation to acetaldehyde in the determination of aspartic acid was prevented. Aspartic acid clearance was much improved after transfusion, although elevated amino acid nitrogen level persisted. Production of lactic from aspartic acid occurred in all phases of shock. This could occur by deamination and decarboxylation of the free carboxyl group.

Amino acid mixture tolerance tests (six dogs in irreversible shock). In the *normal state* three of four dogs showed good blood disappearance curves in one hour. The fourth exhibited incomplete clearance (fig. 9). How much of the amino acid was incorporated into protein cannot be stated. In one of these dogs (Exp. 30) a very large dose of amino acid mixture (2 grams/kgm.) raised the blood amino nitrogen level ninefold, yet the starting concentration was almost regained at the end of an hour. Since only a slight elevation in urea was observed, it may be concluded that protein synthesis or amino acid storage took place. During the next hour injections of 200 mgm. per kgm. were given at six-minute intervals (this data not included in the table). The amino acid nitrogen level rose steadily to the high level (66 mgm. per cent) which the first single tenfold dose produced. The elevation in blood urea was again comparatively slight.

In the *oligemic phase* of shock the tolerance curve was fair in four and poor in one dog. Urea synthesis occurred in two. In one animal the usual elevation in amino acid nitrogen seen in shock did not occur before or after therapeutically

ineffective transfusion. In the two dogs receiving massive doses of amino acid the tolerance curve was fair, but preinjection levels were not reached even two hours later. Urea synthesis was small and out of proportion to what would be expected from the quantity of amino acid cleared. This is interpreted as indicative of protein synthesis or amino acid storage, since urinary excretion of urea or amino acid can be considered negligible under these conditions.

Following *transfusion* good disappearance curves of blood amino nitrogen following administration of the amino acid mixture were obtained in two dogs, despite their irreversibility. The clearance was fair in two dogs. Urea synthesis was noted in three dogs. In one irreversible dog receiving a massive dose of amino acids, amino acid clearance was fair and slow, but urea accumulation was too small to account for the amino acid removed.

COMMENT. The injection of an amino acid mixture, especially in large doses, before and during shock may be followed by some urea synthesis, but not enough to account for the disappearance of injected amino acid nitrogen. It appears, therefore, that protein synthesis takes place in the normal dog and to some extent in shock.

In the normal animal the injection of amino acid mixtures in repeated fractional doses resulted in accumulation of blood amino nitrogen to levels as high as those reached by a full dose given in one injection. The fact that repeated doses of a certain size and given at certain intervals can result in progressive rise in the level of blood amino acid nitrogen in the normal dog suggests that a steady, increased production of amino acid by tissue breakdown, if great enough (20–30 mgm/kgm/min.), could result in persistently elevated amino acid levels without postulating liver failure.

SUMMARY OF RESULTS

Seven naturally occurring amino acids (three as racemic mixtures) were injected individually into dogs in the normal state during the oligemic phase of hemorrhagic shock and following transfusion. In addition to alanine and glycine, acidic, basic, sulfur-containing and aromatic amino acids were studied, as was also an amino acid mixture. The experiments show that the normal animal does not clear the blood of the various amino acids with equal facility. In hemorrhagic shock the clearance of different amino acids varies even more. In general all the amino acids studied are removed from the blood more efficiently following transfusion than during the oligemic phase of hemorrhagic shock. Even when the transfusion is therapeutically ineffective, there is little or no impairment of the ability to clear the blood of the injected amino acids.

Urea production was observed in all phases of shock as well as in the normal state. The urea levels were not as high in the normal state as in shock, presumably because of good renal function in the former.

DISCUSSION

Although the removal of accumulations of amino acid nitrogen consequent to administration of individual amino acids is to some degree slowed during the oligemic phase of shock, presumably as a result of retarded deamination, the fact

that it becomes normal or near normal promptly after transfusion indicates that irreparable damage to the deamination mechanism has not occurred.³ If deamination were in fact deficient because of injury to enzyme systems, the formation of urea should not occur or should be curtailed. The increases in blood urea which we observed are too great and too sudden to be explained on the basis of decreased renal function. Decreased urea synthesis, reported by Engel (7) following the injection of an amino acid mixture in the oligemic phase of hemorrhagic shock in nephrectomized rats, is not conclusive evidence of loss of the deaminating function since it may reflect a decrease in blood flow through the liver, a vital organ in deaminating activity. The evidence he cites (7) to support his conclusion that there is a true metabolic injury, not just a deficiency in blood flow, is derived from *in vitro* work. Unless the injury he postulates is reversible, his conclusion is contradicted by our observation of recovery of the normal rate of deamination after transfusion, as evidenced by the comparison with the rate of amino nitrogen clearance from the blood in the normal state.

Even though the general trend of protein metabolism in shock is catabolic, protein synthesis may occur. For example, in one experiment in which an amino acid mixture was injected in shock, blood clearance was as good as in the normal state (fig. 9). Although the clearance was slower when ten times as much was injected than with the smaller doses, the amount of urea produced as estimated from blood levels above was too small to account for the amount of amino acid cleared. It is likely that protein synthesis occurred. The negligible rise in urea cannot be attributed to excretion via the kidneys, since no urine is formed at a blood pressure of 30 mm. Hg.

If the poorer blood amino nitrogen clearance noted in the oligemic phase of shock is referable to diminished blood flow, especially in the liver, it is difficult to explain the persistence of a normal rate of clearance after the return of peripheral vascular collapse in the posttransfusion phase. Perhaps even under such conditions the flow of blood through the liver is better than in the oligemic phase. Such flow might be sufficient to maintain the function of deamination, even while the process in the liver which is responsible for progressive vascular failure is not favorably affected; thus death may occur before the return of a slower rate of deamination can be observed. The cross-circulation procedure which corrects a failing peripheral circulation after therapeutically ineffective transfusion requires a substantial interval to reverse the hepatic injury involved (18). All liver functions need not be equally sensitive to the injury from anoxia or equally responsive to therapy.

Why does blood amino nitrogen remain elevated after transfusion even though the injected amino acid may be cleared at a normal rate? Possible explanations are a) persistence of normal amino acids so far not studied, b) the formation of

³ Although only one fifth of the possible amino acids involved were tested, we believe that the data are applicable to most of the amino acids, since the ones selected may be considered representative of most types. Three of these amino acids were racemic mixtures of the natural and unnatural forms. However, since both forms were deaminated, interpretation of the results remains valid.

abnormal amino acids which possess the alpha amino carboxylic acid group and which cannot be metabolized but would be measured by the analytical methods employed, c) defective protein catabolism with the release of proteoses or peptides which are not removed by protein precipitation and which might be measured by the analytical methods employed and d) accumulation of a nitrogenous nonamino acid substance measured by the relatively nonspecific colorimetric method (11). In support of the last interpretation is the evidence (19, 20) that amino nitrogen accumulation in the blood during shock is less when measured by the ninhydrin gasometric method (21) than by the colorimetric method employed in this investigation (11).

In some experiments the injection of cysteine, lysine or tyrosine seemed to suppress the usual progressive rise in total amino acid level in shock. However, in one experiment not reported above, injection of a mixture of lysine, cysteine, tyrosine and tryptophane in irreversible shock failed to lower an elevated amino acid nitrogen level while the injected amino acid nitrogen was cleared from the blood normally.

The accumulation of blood amino nitrogen in shock may be related to disturbance in carbohydrate metabolism because, as has been pointed out in a previous publication (1), the injection of pyruvate in shock lowered the elevated blood amino nitrogen concentration in six of nine experiments (see fig. 11). The result could not be due to transfer of amino nitrogen to pyruvate, but could result from conversion of amino acid nitrogen to ammonia by pyruvate. That this is a possible reaction is suggested by an interesting observation of Greenstein (22). He showed that digests of glutamine with liver extracts yield very little ammonia, but when pyruvate was added, considerable ammonia was produced. He ascribed this to the condensation of glutamine with pyruvate to form the dehydropeptide, gamma glutamyl amino acrylic acid, which, when split by dehydropeptidase, gave glutamic acid, ammonia and pyruvic acid. The pyruvic acid served as a cosubstrate.

Engel (23) reported that in the rat there is a correlation between the level of the plasma amino acid nitrogen and the severity of the shock state, and Kline (24) observed that in the dog there is a correlation between the rate of rise in arterial (plasma) amino acid nitrogen in late shock and survival. From Kline's data it appears that the amino acid nitrogen level merely reflects the progressive disturbance in blood flow as death approaches. He demonstrated that the amino acid output from the limbs, which was considerable early in shock, diminished as shock progressed.

The sharp rise in the arterial plasma amino acid nitrogen in late shock cannot therefore be attributable to greater amino acid production in the periphery. It is more likely due to *decreased overall* amino acid removal by the liver even though more efficient amino nitrogen removal per unit volume of hepatic blood flow was indicated by an increased amino acid difference between portal and hepatic venous blood as shock progressed (24). A decrease in total liver blood flow with concomitant decrease in total amino nitrogen removal by the liver would thus explain rising amino nitrogen in arterial blood during shock despite a diminishing supply from the tissues and efficient amino acid clearance by the liver. This

concept is further supported by direct observations showing a marked decrease in liver blood flow in the oligemic phase of shock (25).

Hence the correlation between blood amino acid nitrogen concentration and death or survival in the oligemic phase of shock does not establish a causal relationship between disordered amino acid metabolism and death or survival from shock. We observed no correlation between the capacity to clear amino acid from the blood and the therapeutic effect of transfusion.

The fact that amino acid nitrogen remains elevated for some time after effective therapeutic transfusion as well as in dogs cured by viviperfusion after unsuccessful therapeutic transfusion (18) supports the view that the amino acid level and the mechanism involved in its regulation are not of fundamental importance in the development of irreversibility or in the course of the posttransfusion phase.

CONCLUSIONS

1. Blood disappearance curves for many injected amino acids, singly or in a mixture, were obtained in dogs in the normal state, in hemorrhagic shock and following transfusion. The amino acids studied individually were glycine, alanine, cysteine, lysine, tyrosine, tryptophane and aspartic acid. Normally the injected amino acids were cleared from the blood within one hour in nearly all instances. In hemorrhagic shock some, although not all, dogs showed poor clearance of certain amino acids. Following transfusion clearance was nearly normal in most cases even when circulatory collapse reappeared. There was no correlation between amino acid clearance or blood amino nitrogen concentration and reversibility to transfusion.

2. Rises in blood urea following amino acid injection were observed in some normal dogs and in some shocked animals.

3. The spontaneous rise in total amino acid nitrogen usually seen as shock progressed seemed less in dogs which had received cysteine, lysine or tyrosine.

4. Even when blood amino nitrogen clearance was good in any phase of shock, the increase in total blood amino acid concentration persisted.

5. The injection of large doses of an amino acid mixture resulted in considerable clearance of the blood of the injected amino acids without an equivalent rise in blood urea nitrogen. This would suggest protein synthesis even in dogs in shock.

6. Evidence is presented to show that slower clearance of injected amino acids during the oligemic phase of shock is due to deficient blood flow and not necessarily to damage to deaminase systems. The ability to clear the blood of injected amino acid was not found to bear a significant relationship to the effectiveness of transfusion or to death or survival.

Acknowledgment for technical assistance is due Miss Gertrude Weinberger, Mrs. Ruth Griffin, Miss Dorothy Kaufman and Mr. Thomas W. Barnett.

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INFLUENCE OF HYPOTENSION ON CORONARY BLOOD FLOW, CARDIAC WORK AND CARDIAC EFFICIENCY¹

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Received for publication January 12, 1948

The effect of a sudden reduction in systemic blood pressure upon coronary blood flow and the functional capacity of the heart is a question of considerable practical importance. Coronary blood flow and arterial blood pressure have frequently been shown to be closely related; our own studies have confirmed this (1). Also a diminution of cardiac output usually accompanies a hypotension (2-6). We are not aware, however, of studies that have attempted to correlate the changes caused by hypotension in coronary blood flow and cardiac work, nor has the effect of such changes upon the cardiac capacity to do work been investigated.

A series of studies of the coronary circulation and the oxygen consumption of the dog's heart made during the past two years (1, 7, 8) has led recently to the development of a method for the measurement of these functions in animals subjected to no abnormality other than light anesthesia (9). By the use of this method, changes in coronary blood flow and cardiac oxygen metabolism can be studied under conditions approaching normal. Observations made during hypotension under such circumstances should reveal changes approximating those that occur in man. The present investigation was undertaken, therefore, to determine the circulatory and oxygen metabolic adjustments of the heart during hypotension.

METHODS

The methods employed in this investigation were the same as reported previously (9). Dogs, weighing 15 to 30 kgm., were anesthetized with pentobarbital sodium intravenously, 30 mgm. per kgm. with 30 mgm. supplements if needed. Anesthesia was maintained in a light plane. Blood samples were collected from a) the coronary sinus through a radio-opaque catheter (#8F or #9F) introduced into the right external jugular vein through a small incision and guided into the coronary sinus under the fluoroscope; b) the pulmonary conus by means of a smaller catheter (#6F) similarly introduced; and c) a femoral artery by means of an 18-gauge needle inserted into the artery. Each of these was connected to

¹ The expenses for this investigation have been defrayed by a grant from the Life Insurance Medical Research Fund to the Department of Pharmacology of the University of Pennsylvania.

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a manifold (10); the arterial system was connected in addition to a mercury manometer for the registration of blood pressure.

Coronary blood flow was measured by the nitrous oxide method (10, 11) as applied to the coronary circulation (9, 12). The inhaled mixture consisted of nitrous oxide 15 per cent, oxygen 21 per cent and nitrogen 64 per cent. The period of inhalation was 10 minutes. Cardiac oxygen consumption was calculated from the coronary arterio-venous oxygen difference and the measured coronary flow. The procedures employed in collecting, handling and analyzing the various blood samples have already been described in detail (9, 10, 11).

Cardiac output was estimated by the direct Fick method. The oxygen consumption was calculated from the measurement of the volume of expired air and the analysis of its gas content by the microtechnic of Scholander (13). The expired air was collected by diverting it with flutter valves attached to an endotracheal tube (with an inflated balloon) into a calibrated Tissot spirometer. The period of collection was 5 to 10 minutes, depending upon the tidal exchange.

Peripheral resistance was calculated according to Poisseuilles Law by the standard formula (14):

$$\text{TPR} = \frac{\text{mean aortic pressure (mm. Hg)} \times 1332}{\text{cardiac output in cc. per sec.}} = \text{Absolute Units}$$

Cardiac work was calculated by the formula $W = PQ$ where P is the mean aortic pressure and Q is the cardiac output. The result is expressed as kgm.M/min. The kinetic energy factor has not been considered (15).

Cardiac efficiency was taken to be the ratio between mechanical work and oxygen consumed, both in heat equivalents. It was calculated as in our earlier experiments (7).

Hypotension was produced by the intravenous injection of tetraethyl ammonium chloride in two experiments and by the subdural injection of procaine hydrochloride in six instances. The procaine was injected through a 19-gauge needle introduced into the subarachnoid space at the second or third lumbar interspace. The drug was dissolved in 2 cc. of sterile saline.

RESULTS

The observations made during the control period in 11 dogs are summarized in table 1. In four experiments (14, 16, 21, and 22) the animals had severe anemia and cannot be regarded as normal; it is noteworthy that in three of these coronary blood flow was unusually rapid. Cardiac work tended to be elevated in all cases, chiefly because the cardiac output was high (average 4.2 L/sq. meter/min.). The values for cardiac efficiency were higher than those generally appearing in the literature, but this is not remarkable in view of the figures for cardiac output.

The effects of hypotension are summarized in table 2. The amounts of the drugs used to produce the hypotension are also indicated. In the experiments in which subdural procaine was used, there was flaccid paralysis of the hind limbs from which recovery was complete in all but one instance (expt. 14). Autopsy

revealed that the spinal cord of this dog had been injured by the needle, a probable factor in the very low blood pressure which made impossible the collection of a second set of blood samples. There was no way of knowing the level of spinal anesthesia in the other five dogs.

Among the various findings presented in table 2, the following deserve comment. The effect of hypotension on coronary blood flow was uniformly in the direction of a decrease, but in some cases (expts. 16, 18, and 19) the alteration was too small to be significant despite the fact that mean blood pressure and cardiac output had diminished considerably. In three other experiments (8, 21, and 22) in which the changes in coronary flow were large, the decreases in blood pressure and cardiac output were correspondingly great.

TABLE 1

EXPT.	WGT. KGM.	COR. FLOW CC/100 G/MIN.	O ₂ CON- SUMP. CC/100 G/MIN.	OXYGEN VOL. %			MABP MM. HG	CARD. RATE	C.O. CC/MIN.	CARD INDEX	LF. VENT. WORK KGM/MIN.	EFF. %
				Art.	R. Vent.	Sinus						
6	22.9	79	10.6	17.1	14.8	3.7	125	168	5450	6.05	9.5	32.0
8	21.5	63	8.4	15.4	11.2	2.2	140	132	2960	3.42	5.75	33.7
9	24.8	45	5.6	18.3	15.7	5.9	120	170	2040	2.14	3.40	37.0
10	25.2	107	14.4	16.9	13.5	3.4	176	180	4900	5.10	11.95	36.9
12	30.2	98	13.0	16.5	12.4	3.3	132	124	3860	3.55	7.06	19.4
14	20.9	85	7.4	10.4	6.9	1.7	136	144	3740	4.40	7.10	56.1
16	15.0	105	10.7	11.9	9.0	1.7	95	136	2660	3.9	3.50	19.7
19	15.7	72	10.0	17.2	13.7	3.3	121	216	2830	3.94	4.76	37.7
20	17.2	56	6.8	15.8	11.2	3.7	118	150	3170	4.25	5.10	54.0
21	16.1	139	11.1	10.2	7.0	2.2	120	180	3000	4.22	5.0	27.7
22	19.1	136	13.7	11.4	7.7	1.3	120	150	4570	5.71	7.6	34.6

Cor. flow = coronary blood flow in cc/100 g. lf. ventricle/minute. O₂ consump. = oxygen consumption in cc/100 g. lf. ventricle/minute. Art. = arterial. R. vent. = right ventricle. Sinus = coronary sinus. MABP = mean arterial blood pressure. Card. rate = cardiac rate. C. O. = cardiac output in cc/minute. Card. index = cardiac output in liters/sq. meter of body surface area/minute. Lf. vent. work = left ventricular work in kgm.M/minute. Eff. % = cardiac efficiency.

Left ventricular oxygen consumption varied directly with coronary flow. This was to be expected since the oxygen content of coronary venous blood was already so low in the control period that a significant decrease in the volume of blood flow could not be effectively compensated for by a further widening of the arteriovenous oxygen difference (16). In the experiment in which coronary blood flow decreased least (#19), such compensation proved adequate and the oxygen consumption was not reduced.

Cardiac efficiency was decreased during the hypotension in every case. The correlations of cardiac efficiency with the factors entering into its derivation were: with cardiac output, $r = 0.65$, $p > 0.001$; with cardiac work, $r = 0.61$, $p > 0.001$; with mean blood pressure, $r = 0.52$, $p > 0.01$. Cardiac output and mean blood pressure showed a poor correlation, $r = 0.39$, $p > 0.05$.

Hypotension was not associated with a constant directional change in peripheral resistance. In five experiments (5, 8, 14, 18, and 21) the resistance decreased while in the three others it increased.

TABLE 2

EXPT.	WGT. KGM.	TIME	COR. FLOW CC/100 G/MIN.	O ₂ CON- SUMP. CC/100 G/MIN.	OXYGEN VOL. %			MABP MM. HG	CARD. RATE	C.O. CC/ MIN.	CARD. INDEX	PE- RIPH. RE- SIST.	LF. VENT. WORK KGM/ MIN.	EFF. %	
					Art.	R. Vent.	Sinus								
5	20.0	12:35	60	8.0	18.1	11.8	4.8	108	155	1460	1.77	5900	2.20	13.7	
		1:35	Procaine 40 mgm subdural						85	140	1490	1.81	4550	1.76	11.4
		1:45	53	7.74	17.1	12.1	2.5								
8	21.5	10:15	63	8.40	15.4	11.2	2.2	140	132	2960	3.42	3780	5.75	33.7	
		10:45	Procaine 20 mgm subdural												
		11:00	34	3.60	12.2	7.1	1.7	73	130	1690	1.95	3440	1.71	22.9	
14	20.9	11:05	85	7.40	10.4	6.9	1.7	136	144	3740	4.40	2910	7.10	56.1	
		11:45	Procaine 40 mgm subdural												
		11:55			10.5	5.0	1.2	46	150	1780	2.09	2060	1.1		
16	15.0	10:50	105	10.7	11.9	9.0	1.7	95	136	2660	3.90	2850	3.5	19.7	
		11:30	Procaine 20 mgm subdural												
		11:50	98	9.4	10.7	5.6	1.3	72	182	1540	2.26	3750	1.54	10.1	
18	18.2	11:10	72	11.3	17.9	11.6	2.1	88	168	1770	2.29	5060	2.16	10.7	
		12:05	Etamon 40 mgm intravenously												
		12:10	67	10.1	17.0	9.0	1.8	71	174	1210	1.52	3980	1.20	7.15	
19	15.7	10:50	72	10.0	17.2	13.7	3.3	121	216	2830	3.94	3350	4.76	37.7	
		11:45	Procaine 30 mgm subdural												
		11:55	69	10.2	16.6	11.0	1.8	95	168	1370	1.91	5540	1.81	13.8	
21	16.1	10:50	139	11.1	10.2	7.0	2.2	120	180	3000	4.22	3200	5.0	27.7	
		11:30	Procaine 30 mgm subdural												
		11:45	56	4.83	9.7	5.3	1.1	70	115	1840	2.59	3040	1.80	22.7	
22	19.1	10:15	136	13.67	11.4	7.7	1.3	120	150	4570	5.70	2110	7.60	34.6	
		11:00	Etamon 76 mgm intravenously												
		11:05	85	8.24	10.8	6.2	1.1	89	120	3100	3.88	2290	3.83	19.8	

See table 1 for explanation.

To aid in evaluating these findings it was necessary to know the effects of the withdrawal of the amount of blood required for the analyses (for each nitrous oxide determination 65 cc., for each cardiac output determination 15 cc.). This was done satisfactorily in two experiments which are summarized in table 3. While mean arterial blood pressure did not fall appreciably in either experiment, cardiac output declined in both, though more rapidly in one than in the other. Coronary flow changed slightly between the first and second determinations but

it diminished considerably by the time of the third. Cardiac oxygen consumption followed the same course as coronary blood flow. Cardiac efficiency in these experiments, however, did not follow either the cardiac work or the cardiac output but tended to remain constant between the second and third periods; in experiment 12 it actually did not change significantly at any time.

The effect on cardiac output and cardiac efficiency of the experimental procedure per se, irrespective of the hypotensive measures, must be kept in mind as one interprets the results. These control observations may indicate that the hypotensive alterations were exaggerated.

TABLE 3

EXPT.	WGT. KGM.	TIME	COR. FLOW CC/100 G/MIN.	O ₂ CON- SUMP. CC/100 G/MIN.	OXYGEN VOL. %			MABP MM. HG	CARD. RATE	C.O. CC/ MIN.	CARD. INDEX	PE- RIPH. RE- SIST.	L.F. VENT. WORK KGM/ MIN.	EFF. %
					Art.	R. Vent.	Sinus							
10	25.2	11:00	107	14.40	16.9	13.5	3.40	176	180	4900	5.1	2860	12.0	36.9
		11:50	112	15.30	16.8	12.1	3.07	172	180	3180	3.3	4310	7.5	24.9
		12:35	78	10.10	15.7	9.3	2.83	165	180	2320	2.4	5680	5.3	26.0
12	30.2	11:00	98	13.00	16.5	12.4	3.26	132	124	3860	3.6	2690	7.1	19.4
		11:50	80	12.40	18.8	14.5	3.31	135	182	3380	3.1	3210	6.3	17.7
		12:30	69	10.60	18.7	12.8	3.0	124	160	3140	2.9	3200	5.4	18.3

See table 1 for explanation.

DISCUSSION

Before one can discuss the effect of hypotension on coronary blood flow and oxygen metabolism, it is necessary to stress again that the values for cardiac output, work and efficiency were unusually high in these experiments. The latter two factors depend directly on the first factor and this was indeed high when compared with the values reported by some other investigators (see Wiggers, 14). However, we have made every attempt to make the results reliable. The fact that the mixed venous blood was obtained from the pulmonary conus and that the catheter was not moved between determinations should obviate most of the usual sources of error (17). Careful checks of the system for measuring and analyzing the expired air have revealed no flaws. We therefore believe our results to be reliable.

The most probable reason for the cardiac output being high is the pentobarbital anesthesia, which is known to have the following effects on the cardiovascular system: *a*) tachycardia due to depression of vagal influences on the heart (18, 19); *b*) peripheral vasodilatation (20, 21); and *c*) a rise in mean arterial blood pressure due to an elevated diastolic pressure (20). If general vasodilatation is accompanied by a raised mean arterial blood pressure, cardiac output must be increased. An increased cardiac output and mean blood pressure imply an increased cardiac work. Bazett (22) considers the cardiovascular changes associated with pentobarbital anesthesia similar to those occurring in an animal en-

gaged in mild muscular exercise. The relatively high values for cardiac output, work and efficiency reported herein when viewed in this light are therefore not remarkable.

With regard to the original purposes of these experiments, one of which was to afford an insight into the effects of hypotension upon the functional capacity of the heart, we have been impeded by the limitations of the criteria available for estimating this factor quantitatively. Coronary blood flow was decreased during hypotension in every case, the average being 25 per cent. At the same time, cardiac efficiency declined just as consistently, the average being 36 per cent. However, it would be wrong to conclude from this that the decrease in coronary flow was responsible for a deterioration in the functional capacity of the heart. This is evident from the fact that cardiac work was reduced an average of 56 per cent because mean blood pressure diminished 29 per cent and cardiac output 42 per cent. The oxygen requirements, in terms of work to be done, therefore were reduced to a much greater degree than the oxygen supply (determined by the coronary flow). We have already demonstrated (7) that the oxygen requirement of the heart is one of the principal determinants of coronary flow. Since in the hypotensive experiments, mean coronary flow decreased only 25 per cent while left ventricular work was reduced 56 per cent, it follows that the coronary flow became more abundant relative to the cardiac needs. The concomitant decrease in efficiency is misleading because it was associated with a relatively excessive coronary flow. However, no other single criterion appears to be better able to indicate the change in the cardiac capacity for work.

The term 'cardiac efficiency' is widely employed as a synonym for the ability of the heart to perform its functions. A brief consideration of the formula used in calculating cardiac efficiency shows that it is not this at all (23). Efficiency here is used in its true mechanical sense referring to the work done by the machine divided by the energy put into it. In the heart, however, oxygen metabolic changes occur which are not associated with the performance of work. These changes are as a result of two factors which are as follows.

(a) *'Resting' or 'maintenance' energy utilization.* Any living cell requires a certain amount of free energy simply to maintain its living status, regardless of the nature or degree of its activity. In the case of nerve cells, which do no work (measurable in physical or chemical units), there is at present no means for separating this category of energy utilization (oxygen consumption) from that involved in the performance of a specific function, although the fact that cerebral oxygen consumption runs parallel with cerebral functional activity in animals (24) and in man (11) indicates that some such separation exists. In the heart the situation is somewhat clearer but again there is no separation of the two categories of energy utilization. The significance of this may be gained from figure 1 which summarizes our findings for left ventricular oxygen consumption and left ventricular work in 162 observations representing 49 experiments by either the bubble flowmeter or coronary sinus catheterization technics. It is notable that in no instance did left ventricular oxygen consumption decrease below 2.87 cc/100G/minute even though left ventricular work fell to the negligible figure

of 0.16 kgm.M/minute. This value for oxygen consumption cannot be called 'resting' because the average heart rate was about 140/minute. However, it suggests that a considerable amount of oxygen must be used by the heart solely for 'maintenance', regardless of work done. Variations in cardiac efficiency can therefore be in part dependent upon the proportion of this maintenance oxygen value to the total oxygen consumption (23). If all cardiac work is stopped (e.g., by vagal stimulation) only the maintenance oxygen consumption will take place. Under these conditions, the cardiac efficiency would be nil although the capacity of the heart for work might be great.

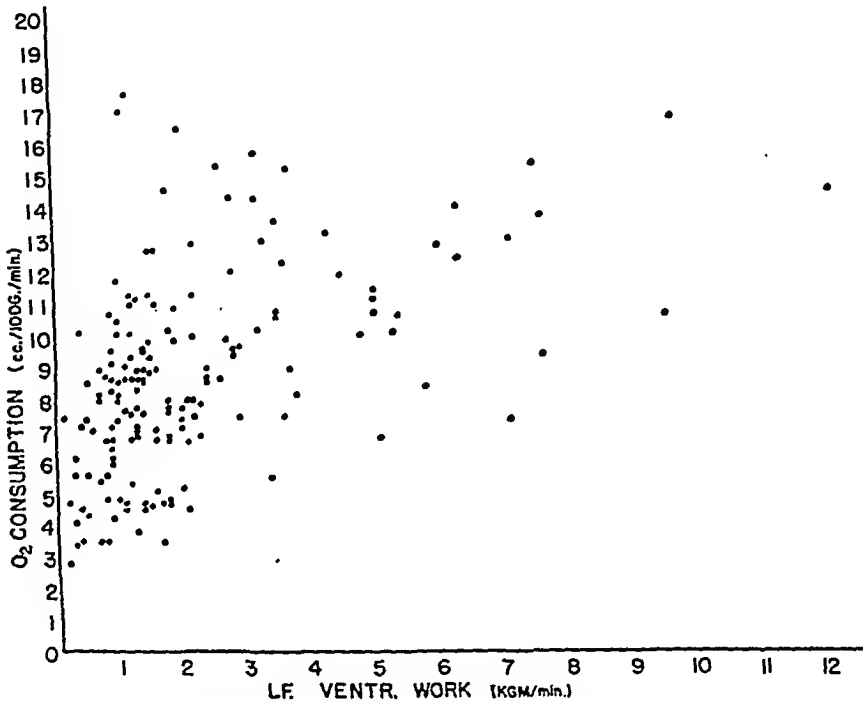


Fig. 1. ORDINATE: oxygen consumption in cc/100 grams of left ventricle/minute. Abscissa: left ventricular work in kgm.M/minute.

(b) *Energy utilization in the isometric phase of ventricular systole.* The energy expended in ventricular contraction up to the point at which intraventricular pressure equals aortic pressure (isometric contraction) does no mechanical work because no blood is moved. The fraction of the cardiac oxygen consumption dedicated to this part of the cycle should thus vary in the same direction as the aortic pressure. If cardiac work is increased primarily by increasing the blood pressure, a relatively excessive amount of oxygen is needed to do the work and cardiac efficiency decreases. That this actually happens has been demonstrated repeatedly (25, 26, 27, 7). Conversely, if cardiac work is raised primarily by increasing the cardiac output, the oxygen requirement is less for the same amount of work done and the efficiency is improved (25, 26, 27, 7). If aortic pressure were raised so high that intraventricular pressure could not be brought to the

same level, no work would be accomplished but the heart would undoubtedly use more oxygen than it would at rest. It is reasonable to assume that the greater the aortic pressure that has to be overcome during the period of isometric contraction, the greater the oxygen metabolism during that period. However, to our knowledge no mathematical method of expression of such relationships has been reported.

Weitz (28) describes Starlings' Law of the Heart (29) as being influenced by, or partly due to, the physical factors that of necessity accompany contraction of circularly arranged fibers. Certainly the relation of the tension of a circular fiber surrounding a sphere to the pressure developed within this sphere is much affected by the radius. However, Weitz accepts the fact that the effect of initial tension on muscular contraction is also involved. No analysis of these complex factors in relation to the work of the heart has been achieved yet to our knowledge.

We believe the conclusion justified that the mechanical conception of efficiency fails to reveal important internal adjustments within the heart muscle during changing conditions.³ It cannot furnish a valid criterion of the condition of the heart or its ability to meet the demands of the work made upon it, since it indicates only the amount of energy used (oxygen consumed) in ejecting a given amount of blood against a given aortic pressure. Of much greater practical and theoretical importance is an understanding of the factors that determine the oxygen requirements of the heart.

Viewed in this light, the effect of hypotension upon the coronary circulation and heart can be summarized as follows. The decline in blood pressure was associated with a diminution in cardiac output so that total cardiac work was greatly reduced. Coronary blood flow was decreased but not to the degree indicated by the low cardiac work. In these dogs with presumably normal coronary arteries, there was no indication that such a hypotension was harmful to the heart. We do not feel that the decline in cardiac efficiency was an indication that the heart was less able to accomplish the work demanded of it.

SUMMARY

1. In the intact anesthetized dog with coronary blood flow measured by the nitrous oxide method, hypotension was produced by the subdural injection of procaine hydrochloride or by the intravenous injection of Etamon.

2. The fall in blood pressure was associated with a diminished cardiac output in seven out of the eight experiments with a consequent marked reduction in cardiac work.

3. Coronary blood flow decreased in all experiments but remained relatively high in view of the marked decline in cardiac work.

³ This would not invalidate statements we have made previously in regard to nikethamide reducing cardiac efficiency (8). In the experiments there cited, nikethamide caused a lowering of the blood pressure, an increased cardiac output and increased cardiac work, all factors that tend to increase cardiac efficiency. In spite of this, the calculated efficiency of the heart was decreased after nikethamide was administered.

4. Cardiac efficiency was reduced in all experiments. There was no evidence that the decline in efficiency indicated that the heart was less able to accomplish the work demanded of it.

5. Evidence is presented that cardiac efficiency is not a valid criterion for indicating the cardiac capacity for work under changing conditions.

The authors wish to express their appreciation to Doctors Carl F. Schmidt, Henry C. Bazett and Robert D. Dripps for their interest and suggestions during this investigation. They also wish to acknowledge the technical assistance of Mrs. Clara Belle Rhode and Miss Hannah T. Broomell.

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ARTERIAL PRESSURE PULSE WAVES IN A PATIENT WITH COARCTATION OF THE AORTA

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Received for publication January 29, 1948

Studies of arterial pressure pulse waves in patients with coarctation of the aorta recorded by means of Hamilton intra-arterial manometers in arteries of the upper and lower extremities have clearly indicated that the diastolic pressure is about the same above and below the coarctation, the systolic pressure is higher above the coarctation and that the femoral pulse lags about 0.1 second behind the radial pulse (1, 2). There is no published record of an arterial pressure pulse tracing from a collateral artery which connects the arterial tree above the coarctation directly with that below. Such a tracing was obtained with a Hamilton manometer in a middle-aged white male suffering from coarctation of the aorta.¹ In addition, arterial pressure was recorded simultaneously on the left side in the radial and femoral arteries (fig. 1). It will be noted that the arterial pressure in the radial artery is 215 to 225/100 mm. Hg, in the left collateral subscapular artery 215/100 mm. Hg, in the femoral artery 150/100 mm. Hg. The mean arterial pressure is 104 mm. Hg in the femoral artery, 110 mm. Hg in the radial and collateral arteries and the pulse wave in the femoral artery lags 0.1 second behind that in the radial artery.

Of greater interest than these usual differences is the remarkable similarity between pulse waves from the radial artery and subscapular collateral artery in spite of entirely different anatomical arrangements of the arterial tree distal to the points from which pressure was recorded. At postmortem examination it was shown that the radial arteries were of normal origin and distribution. Enlarged, tortuous subscapular arteries whose lumina measured 0.5 cm. joined enlarged fifth and sixth intercostal arteries, the lumina of which measured 0.5 cm. and 0.4 cm., respectively. These latter vessels entered the aorta below the coarctation. The main collateral arteries whose lumina measured 0.7 cm. and 0.5 cm. joined enlarged third and fourth intercostal arteries just below the coarctation. The lumen of the aorta was constricted to 0.5 cm. The ligamentum arteriosum was non-patent.

Since variations in the form of pressure pulse waves are considered to be influenced partly by waves reflected from distal arterioles and to a lesser degree by standing arterial waves (3, 4), the similarity of the waves at the radial and collateral arteries is hard to understand. The pulse wave in the radial artery is of a form generally considered to be more representative of the central pulse but

¹ These studies were carried out in 1940.

why this is so is not clear. It suggests that local physical conditions in the segments of the arteries from which pressure is recorded are important in determining the contour of the waves and that in this instance they were the same.

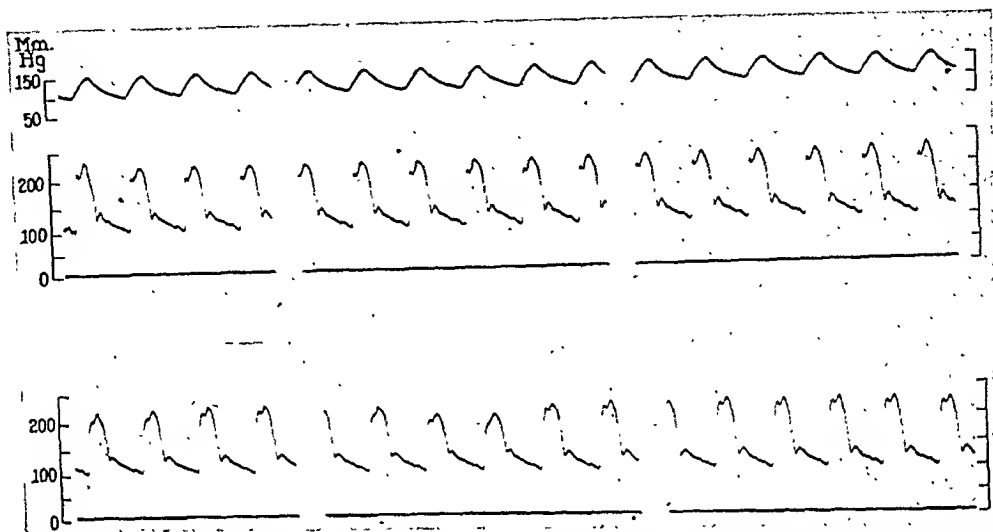


FIG. 1. SIMULTANEOUS RECORDS OF PRESSURE in the left femoral (*upper curve*) and left radial arteries (*middle curve*) taken with Hamilton intra arterial manometers. *Lower curve*, pressure in left subscapular artery recorded a few minutes later.

CASE REPORT

During childhood the patient had occasional convulsive seizures but was otherwise well until the age of 36 (1923) when convulsions began to recur almost weekly, during one of which he developed right hemiplegia and motor aphasia. He was placed in a hospital at the age of 39 (1926) and remained there until his death. On admission to the hospital in 1926, he was noted to have right spastic hemiplegia, Horner's syndrome on the right side, motor aphasia and an arterial pressure of 155/100 in the left upper extremity. Encephalographic studies exhibited atrophy of the left side of the brain and external communicating hydrocephalus. The diagnosis on transfer to Goldwater Memorial Hospital in 1939 was epilepsy, right hemiplegia, and right Horner's syndrome.

It was shortly recognized, by the presence of extensive collateral arteries over the chest and neck and feeble, delayed femoral arterial pulsations compared to pulsations in the radial artery, that the patient was suffering primarily from coarctation of the aorta. The diagnosis was confirmed by angiocardigraphic studies² which demonstrated a constriction in the aorta at the level of the sixth thoracic vertebra. Roentgenograms of the chest exhibited scalloping of rib margins and enlargement of the heart to the left. Until his death in this hospital, the patient experienced frequent bouts of unexplained fever, convulsive seizures,

² These were performed by Dr. Henry K. Taylor, Chief, X-ray Department, Goldwater Memorial Hospital.

short periods of incarceration of a left inguinal hernia, hypochromic anemia which responded to iron therapy and suppurative pneumonia in 1945.

The systolic level of arterial pressure in the upper extremities was generally 180 to 150 mm. Hg and the diastolic 100 to 80 mm. Hg until 1946 when the systolic rose to 190 to 170 mm. Hg and the diastolic fell to 60 to 30 mm. Hg. An aortic diastolic murmur was heard consistently for the first time in 1944. Urea clearance and phenolsulphonephthalein excretion tests of urine function last studied in 1945 were normal. Several cultures of the urine and blood were sterile. In June 1946, he developed heart failure and required digitalis. On February 13, 1947 tonic and clonic convulsive seizures occurred on the left side for the first time and he became unconscious and died. He was 60 years old at death.

Post-mortem examination showed, in addition to coarctation of the aorta and extensive collateral arteries, a congenital aneurysm and compression stenosis of the left middle cerebral artery, extensive encephalomalacia, particularly of the left side of the brain, fusiform aneurysms of the common iliac arteries and a sacular aneurysm (3 cm. diameter) of the abdominal aorta just proximal to its bifurcation.

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ARTERIAL HYPERTENSION IN THE CHICKEN¹

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Received for publication January 19, 1948

For some time we have been interested in the production of hypertension in the chicken in order to study the interrelationship between high blood pressure and atherosclerosis. In our preliminary studies on the chicken we attempted to produce hypertension by the Goldblatt principle by ligation or partial constriction of the main renal arteries (1). In some of these animals after partial constriction of the renal arteries on one side, the ureter of the opposite kidney was ligated in order to increase the ratio of ischemic to normal kidney tissue (2). Unilateral and bilateral obstruction of the ureters was also tried. However, none of these measures consistently increased the blood pressure for any significant period. Even when both ureters were occluded and acute bilateral hydro-nephrosis was produced, a condition which consistently causes a rise in pressure in mammals (3), no increase in pressure was seen in the chickens and the animals died in 12 to 15 hours in acute hyperuricemia (4).

Selye (5) observed that cardiovascular and renal changes could be produced in chickens by adding NaCl to the drinking water of the animals. The initial changes observed were generalized anasarca, cardiac dilatation and renal alterations resembling subacute glomerulonephritis. Later, dehydration, cardiac hypertrophy and glomerular sclerosis were the outstanding features of this salt intoxication syndrome. These changes suggested the possibility that the animals had developed hypertension, but blood pressures were not reported. The possibility that hypertension had been produced seemed worthy of further investigation. After the present studies were begun, Krakower and Heino (6) published data on the blood pressure of chickens on a salt diet which also suggested that a rise in blood pressure occurred. Our results with methods differing from those of Krakower, but somewhat similar to those of Selye, are presented in this report.

METHODS

Eight white leghorn chicks, six weeks old, were placed on a starting diet of mash and tap water for 16 days. During this period three control blood pressures on each chicken were measured with the Hamilton manometer by means of a small needle inserted into the sciatic artery. The drinking water was then replaced by a 0.9 per cent NaCl solution and the chickens permitted to drink this salt solution ad libitum. Blood pressures were taken every five to eight days. After 33 days, when the rise in pressure was seen to be moderate, the NaCl con-

¹ Aided by the Sam Ehrlich Memorial Club Fund for Cardiovascular Research.

² The Department is supported in part by the Michael Reese Research Foundation.

centration was increased to 1.2 per cent. The salt solution was replaced 26 days later by tap water for 13 days. A second period of salt administration (1.2 per cent) was then instituted for 22 days, during which period blood volume, body weight and hematocrit values were determined in addition to the blood pressures. After a final period of 10 days on tap water the chickens were killed.

RESULTS

The systolic and diastolic blood pressures recorded during the experiment in the eight birds are given in table 1, and the average for the entire group is shown in

TABLE 1. EFFECT OF SALT INGESTION ON THE BLOOD PRESSURE OF THE CHICKEN

DAY	FLUIDS GIVEN	CHICKEN NUMBER AND BLOOD PRESSURE IN MM.Hg							
		50	51	52	53	54	55	56	57
0	tap water	122/112	120/105	140/125	130/120	133/116	142/120	140/125	125/115
6	" "	130/119	125/115	150/140	145/135	143/127	142/128	150/135	162/145
12	" "	122/112	120/110	140/133	140/130	134/129	135/125	133/123	140/130
18	salt 0.9%								
22	" "	165/155	125/115	145/133	143/133	130/120	125/115	125/115	136/125
29	" "	158/138	135/125	173/163	155/135	162/142	165/145	160/145	150/135
34	" "			180/165	145/125	140/133		155/136	155/137
42	" "	168/150	143/127	175/155	130/115	155/135		152/138	145/128
50	" "	168/150	150/130	175/160	155/145	145/135		150/140	162/148
51	" 1.2%								
56	" "	143/128	170/127	175/153	173/148	135/120		165/148	162/148
62	" "	183/158	158/134	190/160	180/150			155/138	162/145
69	" "	178/163	168/138	195/167	165/145			145/130	185/163
76	" "	195/170	190/150	190/160	158/138			165/145	205/160
77	tap water								
83	" "	133/120	135/123	135/120				140/125	155/140
89	" "	137/120	128/115	167/147				133/118	165/145
90	salt 1.2%								
99	" "	160/145	148/123	170/155				175/157	168/148
109	" "	165/150	165/150	190/170				175/153	180/160
112	tap water								
121	" "	136/120	155/145	177/163				180/155	160/138

figure 1. The control blood pressure at the beginning of the experiment averaged 132/117 mm. Hg. The values in the several birds did not vary significantly from this value, which is in the range obtained in our laboratory on a large number of controls (7). The high level of the normal diastolic pressure in the chicken appears to be related to its high normal body temperature of 42°C. (7).

At the onset of the 0.9 per cent salt administration the blood pressure did not rise significantly, but at the end of 17 days it had risen 22/17 mm. Hg. It then remained unchanged until the salt concentration was increased to 1.2 per cent. This produced a further increase of 25/9 mm. with the average pressure reaching 183/154 mm. Hg. The total rise over the control values was 51/37 mm. Hg. When the salt was discontinued a prompt fall in pressure occurred, averaging

43/28 mm. Hg and bringing the pressure nearly to the control levels. With the readministration of salt (1.2 per cent) in the drinking water, a rise in pressure was again observed, with a fall after the animals were again returned to ordinary tap water. The weight of five birds during the second period of salt intake showed an average decrease of 330 grams, a 20 per cent reduction over a period of 15 days. Within nine days after the salt intake was ended there was an average gain in weight of 40 grams.

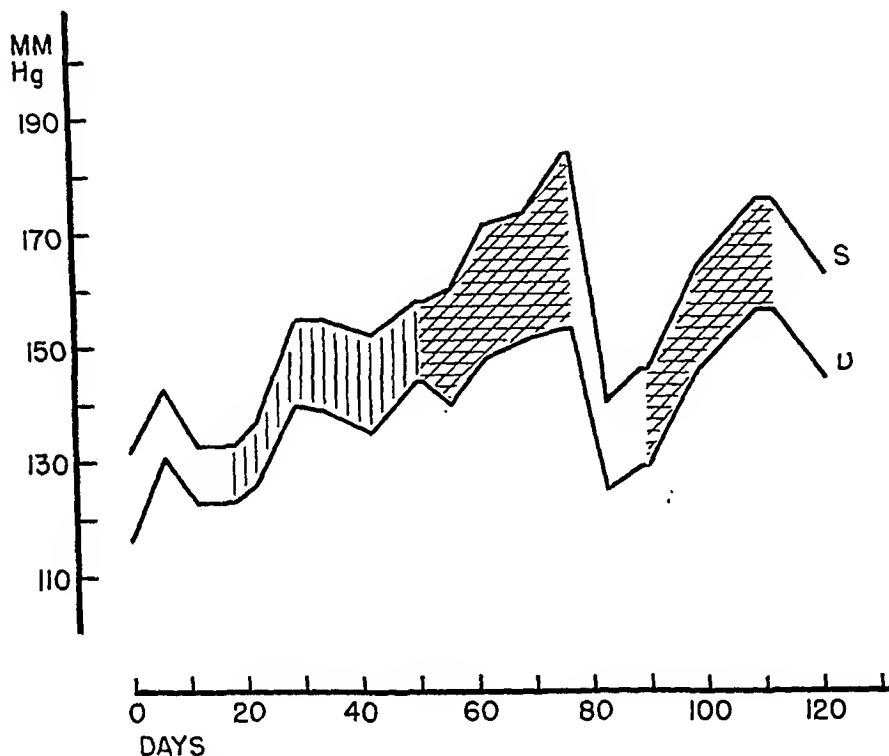


FIG. 1. EFFECT OF INCREASED SALT INGESTION on the average blood pressures of eight chickens. S and D systolic and diastolic blood pressure, respectively. The various periods are indicated by markings between systolic and diastolic pressure lines; periods of tap water ingestion are indicated by blank spaces; 0.9 per cent saline, by vertical-lined period; 1.2 per cent saline, by cross-hatched periods. Ordinates indicate mm. Hg.; abscissae, time in days.

The average hematocrit in these five birds, determined at the same time, showed only a slight increase, from 36 to 39 per cent, during the period of salt administration. Blood volumes measured during the salt intake period in four of these birds showed no consistent deviation from the normal.

The amount of salt solution consumed daily averaged approximately 700 cc. per chicken for the 0.9 per cent salt solution and one liter for the 1.2 per cent concentration. Diarrhea was profuse throughout the period of salt administration. At times the feces were streaked with blood. Obvious anasarca was not observed at any time. No dyspnea was noted. The comb, however, became very pale.

soon after the salt administration was discontinued. A certain lethargy was also noted while salt was being given.

One bird was found dead on the 16th day of the first salt intake period. It showed no evidence of edema or gross abnormalities except for a small pericardial effusion and slight pulmonary edema. A second bird died 10 days after the salt concentration had been increased to 1.2 per cent. It was distinctly dehydrated; moderate pulmonary edema and a pericarditis with a large effusion and thin pericardial adhesions were present; all organs were markedly hyperemic. A third bird was killed for comparison at the end of the first salt intake period. It was dehydrated, had no pericardial effusion and showed no other gross changes.

Gross autopsy findings in the five birds surviving the four-month period were not remarkable. Two birds had slight pulmonary edema. None had any marked effusions. The pericardium appeared normal in all. One bird had small subepicardial hemorrhages in the region of the left ventricle. No other microscopic changes were found in the heart. Only one bird had a possible cardiac enlargement in comparison to the other birds. The ratio of wet heart weight to body weight ranged between 0.45 and 0.60 per cent. This compares with control values ranging from 0.40 to 0.53 per cent (8). Both Selye (5) and Krakower and Heino (6) found cardiac hypertrophy to be a consistent finding in the salt intoxication syndrome. Krakower's figures are distinctly higher than ours for which differing experiment conditions may be responsible. However, he hesitated to attribute the consistent cardiac hypertrophy to a blood pressure elevation, because the hypertrophy occurred regardless of the presence or absence of hypertension.

None of the birds had contracted kidneys. Histological changes were confined to the kidneys. Variations were apparently related to the time of death. Thus, the bird that died during the early part of the study showed no renal changes of note. The kidneys of the bird dying soon after the salt concentration had been increased to 1.2 per cent showed a moderate proliferation of the glomerular tufts and degenerative changes in the tubules. The most pronounced alterations were found in the kidneys of the chicken killed at the end of the first salt intake period. In this bird, there was a marked proliferation of the glomerular tufts with conspicuous hyperplasia of the individual cells and a moderate proliferation of Bowman's capsule. The proliferated glomerular tufts completely filled the capsular space and the capillaries were compressed and ischemic. The kidneys of the five birds killed at the end of the study showed similar changes but to a lesser degree. Proliferation of glomerular tufts and hyperplasia of Bowman's capsule was still evident, but the capsular spaces were not completely obliterated and the capillaries appeared less compressed. Krakower and Heino (6) found glomerular enlargement to be variable in their salt-treated birds, dependent mainly on the normal rate of growth. Inflammatory or sclerotic changes were not encountered in our series. The tubules were for the most part intact or showed a mild cloudy swelling. Arterial and arteriolar changes were likewise absent. It should be noted that in the normal chicken these vessels present a very well-developed muscular coat.

Gastrointestinal hemorrhages were not found at autopsy in our birds, although they may have been present earlier in the course of the experiment, since blood-streaked excreta were observed in several birds.

DISCUSSION

The intake of 0.9 per cent saline by mammals is generally followed by an immediate transfer of this fluid and salt across the circulatory bed into the interstitial fluid. If a large amount of saline is ingested, it may be greater than can be excreted by the unadapted kidney and thus will lead to the development of edema. This is apparently what occurred in the experiments of Selye (5) and of Krakower and Heino (6). In our experiments, a transient questionable edema developed in some animals but all our animals soon lost weight and actually became dehydrated. This may have been due to an adaptation of the excretory function of the animal to the increased load.

Normally the chicken reabsorbs much of the urinary water in the cloaca and excretes only a paste composed of uric acid and other nitrogenous substances. Under the conditions of our experiments, however, the increased fluid intake occasioned by the high salt content of the water results in an apparent overwhelming of the dehydrating mechanism and in a consequent production of a fluid excreta.

The increase in both systolic and diastolic blood pressures during the course of high salt intake is considered significant because the upward trend was present in all of the birds. By comparison, a control series of blood pressures in five normal young chickens taken over a period of 80 days was found to be remarkably constant; the average variation in systolic and diastolic pressures in this group was 8 mm. Hg, with no tendency for the blood pressure to change significantly during the period.

No less striking was the effect of the return to tap water, which produced an average drop in pressure of 43/28 mm. Hg. Since this phenomenon was observed twice, we feel justified in assuming a direct relationship between high salt intake and the rise in blood pressure in the chicken. The mechanism of this phenomenon is not entirely clear. An increased blood volume is probably not involved as our direct measurements show, and as the marked weight loss and dehydration would seem to indicate.

The production of hypertension in the chicken by a high salt intake in the drinking water appears pertinent to the general problem of the genesis of hypertension. It has been known for some time that desoxycorticosterone, which affects the excretion of Na and K, also produces changes in the blood pressure in man and other animals (cf. 9). Particularly is this true in animals on a high salt diet. The low blood pressures seen in Addisonian patients is also associated with a low plasma sodium concentration, and this hypotension can be treated by the oral administration of sodium chloride (10). Further, the recent emphasis on the use of salt-free diets in the treatment of arterial hypertension (cf. 11), which originated primarily on an empirical basis, may be considered to have some support from these and other findings.

The mode of production of the hypertension in the chicken is not clear at the present time. The proliferative renal changes which are observed may begin as an adaptive response to high salt intake, but finally may act to increase the intrarenal pressure and thus decrease the blood flow through the kidney. The relationship of the sodium chloride to the blood pressure changes would, however, emphasize the possibility that the adrenal cortex has an important rôle in the hypertensive process.

SUMMARY

1. The blood pressures of chickens were measured at frequent intervals before, during and after periods of substitution of 0.9 and 1.2 per cent of saline solution for the drinking water. Some determinations of body weight, hematocrit and blood volume were also obtained.

2. The average systolic and diastolic blood pressures increased progressively during the period of salt administration from an average of 132/117 mm. Hg at the beginning of the increased salt intake to a maximum average value of 182/154 mm. Hg. The blood pressure fell promptly to control levels after the withdrawal of NaCl. Similar observations were made during two periods of salt administration and two periods of return to the drinking of tap water. The degree of hypertension appeared to depend upon the concentration of salt in the ingested water.

3. Hyperplasia and proliferation of glomerular tufts and Bowman's capsule with compression of the capillaries were found after prolonged periods of the substitution of saline for drinking water. This hyperplasia is reversible.

4. The relation between the kidney and factors affecting salt metabolism in the production of this hypertension is discussed.

We are indebted to Dr. O. Saphir of the Pathology Department for his critical review of the microscopic sections.

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VALIDITY OF T-1824 IN PLASMA VOLUME DETERMINATIONS IN THE HUMAN

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Received for publication February 13, 1948

Cruickshank and Whitfield (1) in 1945, following experimentation on the cat, stated that a variable portion of initially injected T-1824 was phagocytized by the reticuloendothelial system, thereby 'blocking' that system so that *all* of a second injection of dye would be available for dilution. This concept has since been termed the 'cat effect.' They felt that plasma volume determinations based on single injection dye-dilution techniques would be subject to a possible error up to 20 to 30 per cent. Mather, *et al.* (2) reported the occasional occurrence of the 'cat effect' in humans. Miller (3, 4) experimenting on dogs concluded that the correct interpretation of Cruickshank and Whitfield's results was uncertain. Noble and Gregersen (5) reported that they had never seen the 'cat effect' in dogs or humans studied in their laboratory, although Meneely, *et al.* (6) recently stated that the validity of the blue dye method for estimation of plasma volumes was open to question. It seemed worthwhile to reinvestigate this problem in the human.

We felt it could be assumed that the plasma volume of normal, basal adults while at rest in bed would not change appreciably over a period of 40 minutes (small fluid shifts could be ascertained and corrected for, by estimations of the serum specific gravity) and that two consecutive estimations of plasma volume by identical techniques, 30 minutes apart, should be the same, within the limits of experimental error. If the 'cat effect' was present, however, the first determination should be appreciably higher (dye concentration of the unknown relatively lower) than the second, which would lead to the assumption that there had been destruction, phagocytosis, or removal of a portion of the initially injected dye. Further, we felt that the use of single 10-minute samples (5) for the calculation of plasma volumes, rather than backward extrapolation of disappearance curves, would be justifiable for two reasons. First, the two independent estimations of plasma volume could be made within a shorter period of time, favoring the maintenance of a 'steady state'. Second, presumably the 'cat effect' appears early on the disappearance curves and, if it exists in the human, the 10-minute samples would show the effect adequately.

Accordingly, six young, healthy, adult males, following at least 6 hours of sleep and 12 hours of fasting, were injected intravenously with T-1824 (lot #L6307) purchased from the Warner Institute. The exact amount was determined by a weighed syringe technique and approximated 20 mgm. in each instance. Control samples were taken immediately before injection, and exactly 10 and 30 minutes after injection samples were again removed. A second

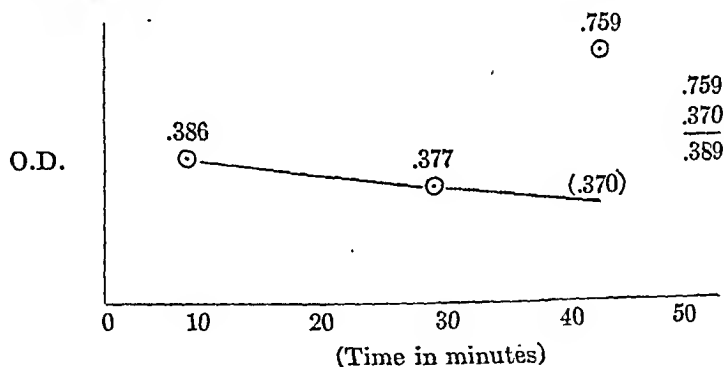
weighed injection of approximately 20 mgm. was made about 2 to 4 minutes following withdrawal of the 30-minute sample. Ten minutes after this injection samples were removed. Samples were taken in duplicate from left and right antecubital veins by separate but simultaneous venipunctures. Precautions were taken to avoid hemolysis and stasis. Timing was done with a stopwatch.

TABLE 1. R. S., 12-16-47

Mgm. dye injected: Injection A—19.074 mgm.; Injection B—19.158 mgm.

	TIME IN MINUTES	ARM	O.D. L620	O.D. L540	O.D. CORRECTED FOR HEMOLYSIS	SERUM SPECIFIC GRAVITY	O.D. CORRECTED FOR HEMOLYSIS AND FLUID SHIFTS
Control	0	L	0	0		(1.0249)	
	0	R	0	0		(1.0249)	
Injection A	0	L					
	10	L	0.382	(.199)	0.383	(1.0247)	0.386
	10	R	0.382	(.196)	0.383	(1.0247)	0.386

	30	L	0.367	(.172)	0.370	(1.0245)	0.376
	30	R	0.370	(.190)	0.372	(1.0245)	0.378
Injection B	32	L					
	42	L	0.739	(.336)	0.743	(1.0244)	0.759
	42	R	0.755	(.381)	0.760	(1.0250)	0.758



$$\text{Plasma volume} = \frac{\text{mgm. injected} \times 0.540 \times 100}{\text{O.D. of unknown}} = \text{Plasma volume A: } \frac{19.074 \times 0.540 \times 100}{.386} = 2670 \text{ ml.}$$

$$\text{Plasma volume B: } \frac{19.158 \times 0.540 \times 100}{.389} = 2661 \text{ ml.} + 15 \text{ ml. (plasma removed)} = 2676 \text{ ml.}$$

Disagreement between A and B: 6 ml. % disagreement between A and B: 0.2%

The samples were allowed to clot and then centrifuged, the serum being removed and recentrifuged. All samples were read directly against the initial dye-free serum in a Coleman Junior Spectrophotometer using a wave length of 620 μ . The optical densities obtained were corrected for imperceptible hemolysis by the method used by Price and Longmire (7). Each sample was then corrected for small fluid shifts by utilizing changes in the specific gravity of the serum,

assuming that the total plasma protein had remained constant throughout the procedure. (The corrections were obtained by applying the following formula:

$$O.D._t \times \frac{S.G._0 - 1.007}{S.G._t - 1.007} = O.D._c$$

in which $O.D._t$ = optical density at time t after the first dye injection; $S.G._0$ = serum specific gravity of the control sample before the first dye injection; $S.G._t$ = serum specific gravity at time t ; $O.D._c$ = theoretical optical density if plasma volume had remained unchanged.) All optical densities, therefore, represent the concentrations of dye that would be present if the plasma volume had not changed during the procedure. The approximate disappearance curve of the initially injected dye, based upon the 10- and 30-minute samples, was projected for another 10 minutes using semi-log paper for the plot. The optical density at this point was subtracted from that of the second 10-minute

TABLE 2

SUBJECT.....		J. G.		H. T.		K. G.		A. T.		W. W.		R. S.	
DATE.....		12-5		12-9		12-10		12-11		12-12		12-16	
ARM.....		L	R	L	R	L	R	L	R	L	R	L	R
Optical densities corrected for hemolysis and fluid shifts	Control	0	0	0	0	0	0	0	0	0	0	0	0
	A.10 min.	.302	—	.311	.310	.311	.314	.314	.317	.296	.296	.386	.386
	30 min.	.290	.291	.300	.301	.302	.302	.306	.306	.285	.285	.376	.378
	B.10 min.	.584	.589	.611	.612	.600	.600	.598	.600	.578	.578	.759	.758
Plasma volume A		3188 ml.		3149 ml.		3184 ml.		3417 ml.		3414 ml.		2670 ml.	
Plasma volume B		3213 ml.		3215 ml.		3220 ml.		3374 ml.		3440 ml.		2676 ml.	
Disagreement		25 ml.		66 ml.		36 ml.		43 ml.		26 ml.		6 ml.	
% Disagreement		0.8%		2.1%		1%		1.3%		0.8%		0.2%	

sample to correct for the residual dye present in the serum and its disappearance from the time the 30-minute samples were withdrawn until the second 10-minute samples were taken. Plasma volumes were then calculated after the method of Noble and Gregersen (5). The volumes calculated from the reinjected dye were also corrected for the plasma removed during the procedure. A representative experiment is illustrated in table 1. Results are summarized in table 2.

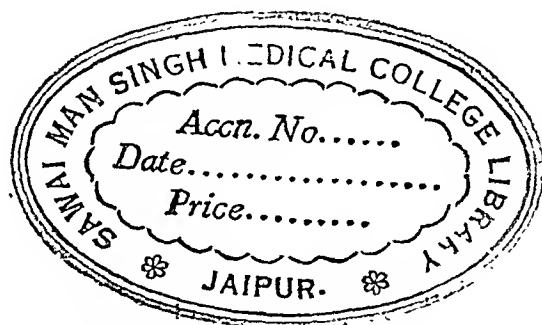
The 'cat effect' was not observed in this series of six consecutive experiments. The close agreement of optical densities of the 10-minute samples, which were taken simultaneously from left and right arms, is further evidence that in the normal, basal human the dye is completely mixed within 10 minutes' time. In our hands the use of T-1824 in the normal, basal human for determination of plasma volume (using a 10-minute sample for calculation of each plasma volume) suggests reliability as shown by the close agreement of optical densities of samples withdrawn simultaneously, and by the checks obtained in repeat estimations of plasma volume within a period of 40 minutes. Although recent work

on dogs (4) questions whether the 10-minute sample actually measures *total* plasma volume, our choice of 10-minute samples in these experiments would still appear to be valid, in that, at least, we are attempting to measure the *same* volume of plasma by each reinjection. Our use of a single 10-minute sample was for the reasons outlined above and even though our calculated plasma volumes may represent the 'circulating plasma volume' (4), we feel that the close agreement of the independently calculated plasma volumes (an average difference of 35 ml. in an average plasma volume of 3170 ml.) enhances the probability that the correct use of disappearance curves of the dye T-1824, with backward extrapolation to zero time, will give even more reliable estimates of total plasma volume.

We wish to thank Dr. E. M. Greisheimer for her immense assistance in this project, and Drs. M. J. Oppenheimer, E. E. Aegerter, Robert Houston Hamilton, J. R. Willson, George Henny and F. W. Sunderman for their continued interest and advice. In addition, we would especially like to thank all of our volunteer subjects, who participated in these and prior experiments, for their time and cooperation. This work was supported in part by RG-194, N. I. H.

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SERUM AC-GLOBULIN: FORMATION FROM PLASMA AC-GLOBULIN; ROLE IN BLOOD COAGULATION; PARTIAL PURIFICATION; PROPERTIES; AND QUANTITATIVE DETERMINATION¹

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Received for publication January 9, 1948

In work on the purification of prothrombin (1-4) it was noted that thromboplastin and calcium ions activated purified prothrombin less rapidly than prothrombin in native plasma (4, 5). This indicated that either the prothrombin molecule was damaged during the purification procedures or that a 'convertibility factor' had been removed. The possibility of damage to the molecule seemed likely because of the ease with which many ordinary laboratory manipulations destroyed the activity completely. A slight change in reactivity was, therefore, regarded as a logical intermediate stage in denaturation. This belief was further strengthened by the fact that heating not only destroys prothrombin but leaves some material of low-grade reactivity (2). Thrombin produced much the same effect on prothrombin (6), and experiments with fibrinolysin gave typically the same picture (7). As a result of these facts, the term *paraprothrombin* was proposed by Seegers (8) as a convenient description of this sluggish reacting prothrombin (7, 8).

The possibility of the existence of a convertibility factor was indicated in several reports (9-11). The prothrombin conversion rate was found to vary from one species to another being especially slow in man and guinea pig (9). Compensatory mechanisms were found to exist in vitamin K deficiency at low prothrombin levels (10, 11), providing for adequate coagulation by increasing the rate of prothrombin conversion. In one report, Smith reasoned that prothrombin activation rate is "determined by a 'convertibility factor' of unknown nature" (12).

Conclusive evidence for the existence of such a factor was obtained independently in three different laboratories from studies with purified materials (13-16). A human case deficient in the factor was described by Owren (17). The accelerator factor has been obtained in concentrated form in two laboratories (16, 18). When small amounts of the concentrates are added to purified prothrombin products, the prothrombin activation rate is invariably as rapid as that found in native plasma. This seems to preclude the possibility of damage occurring to the prothrombin molecule during purification. Consequently there is now no need for the term *paraprothrombin* (7, 8). Attention has been directed to studying the convertibility factor. It accelerates the interaction of pro-

¹ Aided by a grant from the U. S. Public Health Service. Parke, Davis and Co. supplied large quantities of plasma and funds for research. The material in this paper was presented in part before the annual meeting of the Canadian Physiological Society, London, Ontario, October 24, 1947.

thrombin, thromboplastin and calcium ions. Since it is a globulin and an accelerator, it has been referred to by the term 'Ac-globulin' (15). It has been shown by Ware, Murphy and Seegers (19) that a similar, but more potent, accelerator is present in serum. To distinguish between the two it is convenient to speak of plasma Ac-globulin and serum Ac-globulin in accordance with their respective occurrences or origins in plasma or serum.

The following facts were deduced from experiments to be described in this paper. It is probable that plasma Ac-globulin is a pro-enzyme, therefore, being completely inactive. It is partially activated by amounts of thrombin which are too small to cause clotting of plasma. Slightly larger quantities of thrombin completely activate the enzyme and greater amounts, in addition, actually destroy its activity. The chemical properties of serum Ac-globulin are similar to those of its plasma precursor. The methods described herein for the purification and quantitative determination of serum Ac-globulin are fundamentally similar to those already described for plasma Ac-globulin (18).

EXPERIMENTAL

Relative effects of serum and plasma on the activation of purified prothrombin. Blood collected from a single cow was divided into two parts. One part was allowed to clot and the other was mixed with one seventh its volume of a solution of 1.85 per cent potassium oxalate. An hour later the two parts were centrifuged and the activities of the accelerator substances present in the serum and in the plasma were compared at three different concentrations (fig. 1) by a procedure similar to the two-stage method for determination of prothrombin (20-22). The serum or plasma was allowed to act on prothrombin in the presence of an excess of thromboplastin, under standardized conditions, for varying lengths of time; the quantity of thrombin formed was then measured in terms of clotting time determined by the addition of standardized fibrinogen.

In the same concentrations, serum produced thrombin much more rapidly than plasma. Within the limits of experimental error, the final yields of thrombin were the same in each paired experiment. It is apparent that plasma Ac-globulin becomes more active as a result of the blood coagulation processes. However, it is not indicated whether the accelerator is completely activated in serum.

Activation of plasma Ac-globulin. Thrombin is the substance which produces the change occurring in Ac-globulin during clotting (19). Therefore, it is necessary to study the effects of thrombin on oxalated plasma. The plasma was incubated with varying amounts of thrombin (table 1). The thrombin was a very active concentrate of purity comparable to the products described by Seegers and McGinty (23). It is very improbable that, in the dilutions used, other clotting factors were introduced by the added thrombin. The formation of serum Ac-globulin was studied by diluting the mixtures of plasma and thrombin (table 1) with saline and by incubation with a standardized mixture of prothrombin, thromboplastin and calcium ions. By measuring the time required for the thrombin formed to clot fibrinogen, a quantitative concept of thrombin yield was obtained. The results are recorded in table 1 and in figure 2.

One tenth to 0.2 of a unit of thrombin was usually not sufficient to clot the plasma in one hour, but it was adequate to produce serum Ac-globulin in quantities easily detectable. Ten units of thrombin were apparently required to produce a maximum yield of serum Ac-globulin. With 30 or more units of thrombin the resulting serum Ac-globulin was less active and with 200 units of thrombin the activity was reduced by 90 per cent. This loss of activity is believed to be due to the destruction of serum Ac-globulin by thrombin. Preliminary experiments with purified Ac-globulin and thrombin substantiate that view.

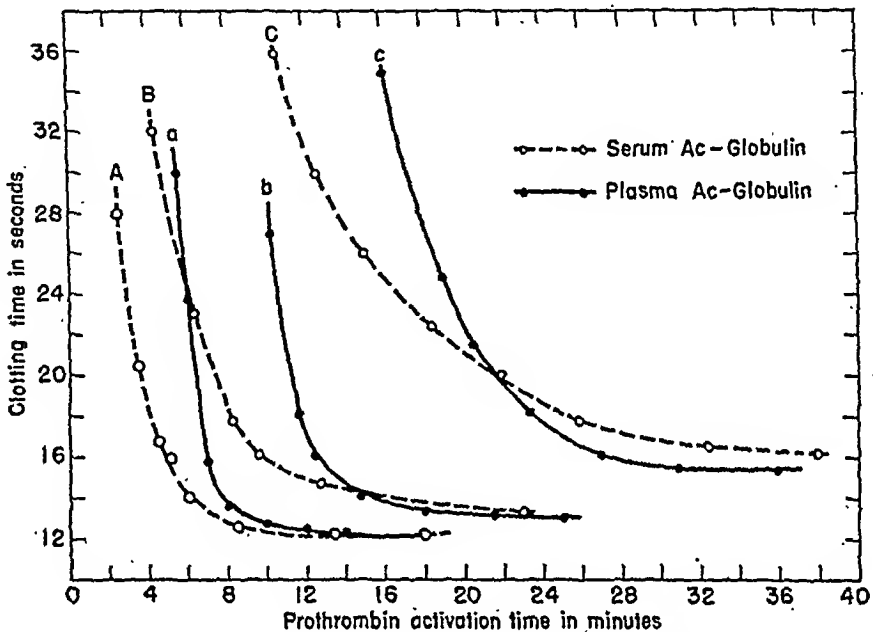


FIG. 1. RELATIVE EFFECTS OF SERUM AND PLASMA ON THE ACTIVATION OF PROTHROMBIN. A and a, serum and plasma, respectively, diluted 1500 times; B and b, serum and plasma, respectively, diluted 4500 times; C and c, serum and plasma, respectively, diluted 20,000 times. Dilutions of plasma include anticoagulant added. The diluted serum or plasma, plus a constant amount of Ac-globulin free prothrombin, were added to the incubation mixture recommended for the two-stage analysis for prothrombin (20-22). Thrombin formation was measured by addition of fibrinogen at intervals. Clotting time is inversely proportional to thrombin concentration.

As a matter of interest it seemed advisable to determine whether fibrinolysin can activate plasma Ac-globulin. In concentrations of 1 to 20 units (8), added to 1 cc. of oxalated plasma, no activation was noted.

Purification of serum Ac-globulin. Work on the purification of serum Ac-globulin was conducted with two objectives in view. One was to find a method for obtaining potent concentrates; another was to test the theory that true serum Ac-globulin is the same as the serum Ac-globulin produced artificially by mixing thrombin and oxalated plasma.

Beef serum was obtained from a bovine blood after it had stood at room temperature for five to six hours. Defibrinated oxalated beef plasma was obtained by

the addition of Thrombin Topical [Parke, Davis and Co., prepared as described (2)] in a concentration of 15 units per cc. to oxalated plasma.² The thrombin was added at room temperature and the fibrin clots were removed as they formed. It was not possible to distinguish between the serum Ac-globulin products obtained from either source. The best yields, with either source material, were

TABLE 1. INFLUENCE OF VARYING AMOUNTS OF THROMBIN ON THE AC-GLOBULIN IN OXALATED BOVINE PLASMA

REACTING CONSTITUENTS		REACTING TIME	PRODUCTS OF REACTION ³
Oxalated Plasma	Thrombin		
cc.	units ¹	hours ²	
1	0	0	No reaction. Tube contains typical plasma Ac-globulin.
1	0.1 to 0.2	1	Serum Ac-globulin always detected. Usually no clot formed.
1	1	1	Formation of some serum Ac-globulin. Partial clot formation.
1	3	1	Large amount of serum Ac-globulin formed. Clot formed.
1	10	1	Complete conversion to Ac-globulin. Clot formed.
1	30	1	Serum Ac-globulin formed and some destroyed. Clot formed.
1	100	1	Serum Ac-globulin formed and considerable portion destroyed. Clot formed.
1	200	2	Serum Ac-globulin formed and 90% destroyed. Clot formed.

¹ Added in a volume of one cc. to give final concentration of thrombin indicated.

² The reaction between plasma Ac-globulin and thrombin to form serum Ac-globulin is rapid. Time was allowed for destruction of added thrombin by anti-thrombin.

³ If clots were present they were removed mechanically and the resulting Ac-globulin was measured by its ability to accelerate the interaction of prothrombin, thromboplastin and calcium ions (fig. 2).

obtained by the method already described for preparation of plasma Ac-globulin in concentrated form (18). The method consists of acid fractionation of diluted plasma or serum, adsorption on magnesium hydroxide suspension, elution with carbon dioxide under pressure, fractionation with cold concentrated ammonium sulfate solution, dialysis to remove salts, and isoelectric fractionation.

² The plasma was oxalated with a special oxalate mixture, low in electrolytes (3).

Serum Ac-globulin of high purity has also been obtained by addition of small amounts of thrombin, one unit per cc. or less, to purified plasma Ac-globulin. This method of preparation is preferred because of its simplicity and because the products are of higher quality than those prepared from either serum or defibrinated plasma.

Quantitative analysis of serum Ac-globulin. Quantitative measurement of activity is made by an adaptation of the two-stage method for determining prothrombin (20-22). When the prothrombin, thromboplastin, calcium ions, tem.

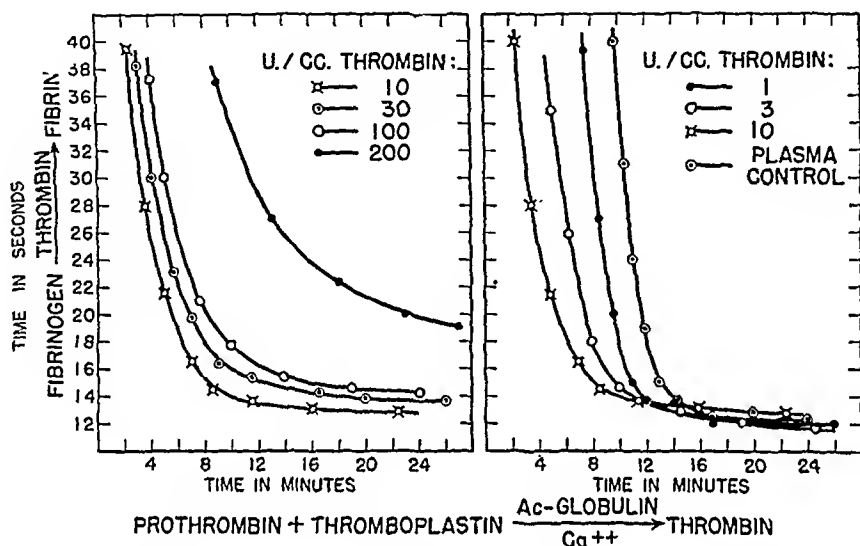


FIG. 2. INFLUENCE OF VARYING AMOUNTS OF THROMBIN ON THE AC-GLOBULIN IN OXALATED PLASMA. The plasma was treated according to the outline in table 1, diluted 5000 times, and allowed to accelerate the activation of standardized prothrombin. Thromboplastin and calcium ions were used according to the specifications of the two-stage analytical procedure for prothrombin (20-22). The thrombin formed in these reactions was measured at intervals by the addition of fibrinogen. The clotting times (ordinate) found at various activation times (abscissa) represent the curves in the figure. Thrombin concentration is inversely proportional to clotting time. The standard prothrombin preparation was Ac-globulin free and in the concentrations used yielded 12-second clotting with fibrinogen when fully activated to thrombin. The curves at the right represent various stages in the formation of serum Ac-globulin from plasma Ac-globulin; those at the left indicate that serum Ac-globulin was formed and partially destroyed by relatively large amounts of thrombin.

perature, salt concentration, pH, etc. are kept constant, the rate of thrombin formation is proportional to the quantity of serum Ac-globulin in the reaction mixture. The curves in figure 3 represent the different rates of thrombin production resulting from varying concentrations of serum Ac-globulin. The curves are similar to those described elsewhere for quantitative determination of the plasma accelerator (18) and they were obtained by the same procedures. In order to determine the concentration of serum Ac-globulin in an unknown, it is first necessary to add a portion of it to the reaction mixture containing standardized prothrombin, thromboplastin and calcium ions. Thrombin formation is

then followed by addition of fibrinogen, at intervals, to portions of the reaction mixture. By plotting the activation curve on figure 3, the concentration of serum Ac-globulin in the unknown can be estimated. If the activation curve of the unknown does not fall between curves 5 and 60 on the figure, a new dilution of unknown should be made because this range has been found to be the most accurate and reproducible portion of the family of curves.

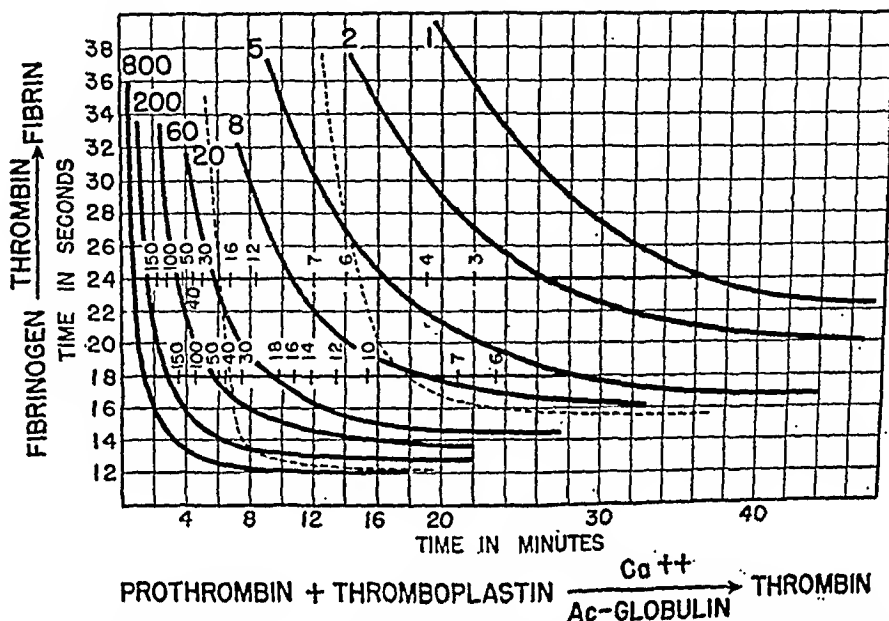


FIG. 3. ACCELERATION OF PROTHROMBIN ACTIVATION WITH VARYING CONCENTRATIONS OF SERUM AC-GLOBULIN. These curves were obtained by adding varying amounts of serum Ac-globulin and a constant amount of prothrombin to thromboplastin and calcium ions under the conditions specified by the two-stage procedure for prothrombin analysis (20-22). At intervals, fibrinogen was added and clotting times were recorded. The amount of thrombin present is inversely proportional to clotting time (ordinate). The prothrombin and thromboplastin were relatively free of Ac-globulin as indicated by the slow formation of thrombin when no serum Ac-globulin was added. Under these conditions, one hour was required to produce enough thrombin to give 40-second clotting with fibrinogen. The prothrombin was of sufficient strength to give 12-second clotting with fibrinogen when fully activated. Concentrations of serum Ac-globulin (units per cc. x 1000) are represented by the large numbers at the tops of the curves and by the small numbers on the 24- and 18-second clotting time coordinates. The latter were derived by interpolation. The broken lines (rectangular curves) are for comparison, representing prothrombin activation curves obtained by the same method with two different concentrations of plasma Ac-globulin instead of serum Ac-globulin.

A unit of serum Ac-globulin is defined as 1000 times that amount which when present in one cc. of the reaction mixture will reproduce curve 1 of figure 3. This unit is approximately of the same magnitude as the arbitrary unit described for plasma Ac-globulin (18). One unit of plasma Ac-globulin should yield approximately one unit of serum Ac-globulin when the former is fully activated. It is unfortunate, however, that a precise correlation cannot be made with the use of data now available.

Stability of serum Ac-globulin. Serum Ac-globulin is remarkably stable in serum. Two samples of beef serum, collected by centrifugation one and one half hours after the blood was drawn, were stored at 5° C. The accelerator activity was compared at intervals with a pooled sample which had been frozen and stored at -30° C. The analytical values indicate, within the limits of experimental error, that no significant loss of activity occurred on storage at 5° C. for a period as long as 26 days (table 2).

TABLE 2. STABILITY OF ACCELERATOR IN BOVINE SERUM AT 5° C

DAYS OF STORAGE AT 5° C.	CONCENTRATION OF SERUM AC-GLOBULIN		
	Serum 1	Serum 2	Control serum ¹
	Units/cc.	Units/cc	Units/cc.
0	115	59	72
8	164	71	87
16	102	65	66
26	90	55	73

¹ Mixture of serum 1 and serum 2, stored at -30° C.

DISCUSSION

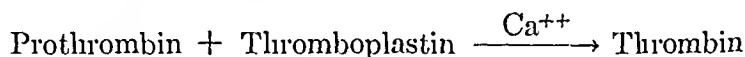
It has been mentioned repeatedly in the literature that in the clotting processes a 'latent period' precedes the period of thrombin production. After a small amount of thrombin is produced, further formation of thrombin apparently results from autocatalysis. The explanation for this phenomenon is furnished by our observations which have shown that Ac-globulin in plasma becomes more active in the presence of small amounts of thrombin (19, 24). Apparently the thrombin hastens its own formation by forming an intermediate (serum Ac-globulin) which, in turn, catalyzes further thrombin production. These reactions are more accurately termed co-autocatalysis (19, 24) instead of autocatalysis.

In order to attempt an explanation of the rôle of Ac-globulin in blood coagulation it is first necessary to mention that thrombin is produced slowly, in the absence of Ac-globulin, by the interaction of thromboplastin, prothrombin and calcium ions. This has been proven in this laboratory by extensive work which will be reported at a later date. This conclusion is in conflict with the recent interpretations presented by Owren (16).

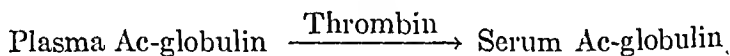
From the prothrombin activation curves of figure 2, it is possible to conclude that plasma Ac-globulin itself does not act as a catalyst. It is a pro-enzyme and must first be changed, by thrombin, to the active catalyst, serum Ac-globulin. In figure 2, the curve at the extreme right represents the interaction of thromboplastin, prothrombin and calcium ions in the presence of plasma Ac-globulin. There was first a latent period, more accurately called a period of slow thrombin production, during which very small amounts of thrombin were formed by the interaction of thromboplastin, prothrombin and calcium ions. This period lasted for about 11 minutes during which time approximately 5 per cent of the prothrombin was converted to thrombin. The remaining 95 per cent of the

potential thrombin was produced during the following two to three minutes. This period of rapid thrombin production results from the action of active catalyst and coincides with the formation of additional active catalyst. The same prothrombin, thromboplastin and calcium ions, alone forming thrombin at a slow rate, required from one to one and one half hours to produce a 30 to 40 per cent yield. When plasma was added which was previously activated with thrombin (10 units per cc.), the period of slow thrombin production was reduced to approximately two minutes. This acceleration of thrombin formation is much greater than was achieved with a comparable amount of untreated plasma. These facts make it very unlikely that active Ac-globulin exists in plasma; if there is any of the active catalyst in plasma, the amount is probably small as compared to the quantity of inert Ac-globulin present.

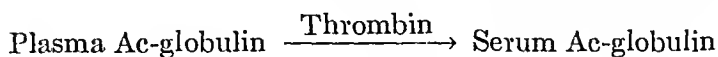
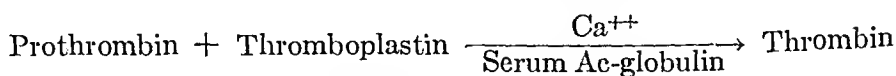
Assuming the above interpretations are correct, it is now possible to attempt a description of the processes of blood coagulation as they occur. The equation below represents the *first* reaction:



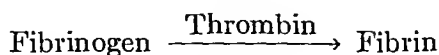
This permits the key concept that *serum Ac-globulin is the result of thrombin being formed first*. This reaction is slow but on the basis of mass law a high plasma prothrombin concentration presents favorable conditions. Perhaps that is why such an apparent excess of prothrombin is provided in plasma. The quantity of thromboplastin is the limiting factor in normal physiological conditions. The above reaction corresponds to the 'latent period' in blood coagulation. Even when 0.1 of a unit of thrombin per cc., about 0.03 per cent of the total potential thrombin of plasma, is liberated some serum Ac-globulin is produced (cf. table 1) as follows:



This reaction is at first slow but as more catalyst (thrombin) becomes available it progresses more rapidly, producing more serum Ac-globulin. Meanwhile no clot has formed. However, the following reactions gain impetus:



The thrombin titer soon rises high enough to cause clotting of fibrinogen as follows:



Thereafter the above reactions become spent. The thrombin formed is destroyed by antithrombin and antithromboplastin acts on the thromboplastin (25-27). Serum Ac-globulin survives, or at least it does in the bovine species. Whether it does in other species remains to be determined.

It is theoretically possible to have serum Ac-globulin circulating in the living organism thus furnishing the individual with a hyperreactive coagulation system more sensitive to the action of thromboplastin than normal. An adequate source of thrombin, for forming the serum accelerator, could be derived from a bruise or from the vicinity of a thrombus. This may be of importance in the problem of thrombosis. It might be suggested that such minute amounts of thrombin would easily be disposed of by the action of plasma antithrombin. However, that is not in accord with the known facts of the reaction kinetics involved. It has been shown that the interaction of thrombin and antithrombin is an equilibrium reaction (28). The last traces of thrombin are removed with extreme difficulty (29) so that it is probably possible for small amounts of thrombin to remain active in the blood for enough time to partially activate plasma Ac-globulin.

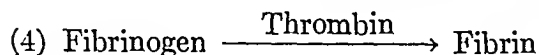
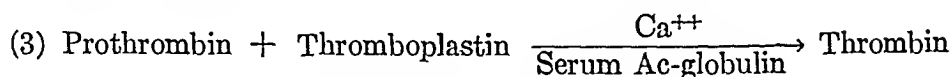
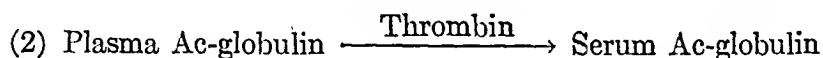
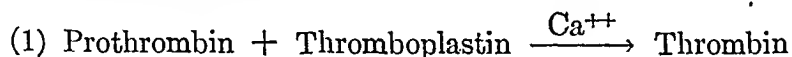
It seems likely that serum Ac-globulin is identical with Factor VI of Owren (16). He concluded that Factor VI is formed from Factor V (plasma Ac-globulin?), but he did not recognize the key rôle of thrombin in this transformation. He, therefore, proposed that 'prothrombin? + Factor V $\xrightarrow{\text{Thrombokinese} + \text{Ca}^{++}}$ Factor VI' and that the latter is the true activator of ? prothrombin. The data reported above show that calcium ions are not necessary for the formation of the active accelerator, and they indicate that serum Ac-globulin is not the true activator of prothrombin. Owren did not recognize the basic difference between the plasma and serum type accelerators. In some experiments he used defibrinated plasma as a source of his Factor V products. He, therefore, used the plasma and serum accelerators interchangeably. It is also of interest that he reported a concentration of Factor V in serum as 190 per cent of that in plasma. This result is explained by the data reported above; serum Ac-globulin is capable of activating prothrombin much more rapidly than plasma Ac-globulin; a qualitative difference was mistaken for a quantitative difference in Owren's experiment.

SUMMARY

Plasma Ac-globulin is probably an inert protein or a pro-enzyme. It is changed to serum Ac-globulin by thrombin. Fibrinolysin will not produce the change. Serum Ac-globulin is the active catalyst of the interaction of prothrombin, thromboplastin and calcium ions. It is present in bovine serum and may be formed in oxalated plasma or from purified plasma Ac-globulin by the addition of small amounts of thrombin. Large quantities of thrombin destroy Ac-globulin activity. Thrombin is formed by co-autocatalysis in the blood-clotting mechanism.

Serum Ac-globulin has been obtained in concentrated form from bovine serum and from defibrinated oxalated bovine plasma. Its chemical properties cannot be differentiated from those of plasma Ac-globulin. Stability studies show that serum Ac-globulin is quite stable in bovine serum. Its activity can be measured quantitatively by the methods described.

The function of Ac-globulin in the blood-clotting mechanism is summarized by the following equations:



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INFLUENCE OF HEPARIN ON THE OPTICAL DENSITY OF CLOTTED PLASMA¹

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Received for publication January 15, 1948.

In assaying heparin by the use of beef and sheep plasma, the problem of assessing the end-point led to a study of the optical density of the clot. The transmission of light as a means of studying coagulation of plasma has been used by various workers (1). The clotting of fibrin in a system of purified components was reported by Ferry and Morrison (2). These studies did not include heparin. Meylink (3) proposed the use of the electrophotometer for assaying heparin but gave no data and stated that the end-point should be taken at corresponding points on the coagulation curves in the region where conversion of fibrinogen is most rapid.

The present investigation was undertaken with the same object in view as Meylink's study, that is, to determine the applicability of the optical method to the assay, and was in progress when Meylink's report became known. An assay could be carried out in two ways: 1) establish time-density clotting curves for different concentrations of each sample and standard and make proper comparisons, or 2) set up the experiment as in the usual assay technique and after one or two hours read the degree of clotting with the electrophotometer instead of by the visual method. The first method is not adaptable for practical assay work because of the limited number of samples that can be run simultaneously, and because the rapid deterioration of plasma obviates quantitative comparison of samples not run concurrently. The chief objection to the second method (and it applies to the first as well) is the lack of uniformity of the clots in many instances, more particularly in beef than in sheep plasma (4). This lack of uniformity can lead to considerable error in determining the light transmission since in practically all photoelectric instruments the light beam passes through only a portion of the medium.

This report covers investigations on time-density clotting curves as determined by the photoelectric method. It is not concerned with assay material as proposed under 2) above and it will be seen that the use of the clotting curves themselves cannot be adapted very well to an assay method. A preliminary report on this study was given at the 1947 Federation meeting (5).

METHOD

Optical density has been expressed in terms of reciprocal function, the per cent transmission of light. Measurements were made with a Fisher electrophotom-

¹ Supported by a grant from the United States Public Health Service.

eter. Thirteen x 100 mm. lipless pyrex tubes selected for uniformity were employed. The tubes with cork stoppers were too tall for the cover of the instrument to close tightly so a section of it was cut out and a light tight hood attached.

The procedure consisted of recalcifying citrated plasma in the presence of varying concentrations of heparin. This was similar to the assay technique (4). Beef and sheep plasma stored in the frozen state were used. The volume was 3 cc. of which 1.5 cc. represented the plasma. A volume of 2 cc., employed in the assay, could not be used on account of the size of the light aperture, and it seemed better to increase the volume rather than alter the instrument. The ingredients were added in the following order: heparin, plasma, saline and calcium chloride. After adding calcium the tubes were immediately stoppered with paraffined corks, inverted four times and placed in the instrument. Time was measured from the moment of inversion. The tests were conducted at room temperature. The red filter was used in the electrophotometer.

The first or 'zero' reading was made at one minute and subsequent readings at appropriate intervals thereafter. Two tubes were run simultaneously. The instrument was first adjusted to read 100 per cent transmission using distilled water as the standard. Plasmas then showed approximately 95 per cent transmission.

Curves of the results were drawn by plotting the per cent transmission against the logarithm of the time in minutes. Quantitative comparisons are valid only between two tubes run simultaneously. Because of gradual deterioration of the thawed plasma all comparisons between tubes run at different times must be considered qualitative.

Species and individual variations of plasma (fig. 1). Coagulation in unheparinized plasma was followed for several hours. The amount of light transmitted was reduced to about 65 per cent in sheep plasma and about 20 per cent in beef plasma. Curves for two samples of each species are shown in figure 1. One sample of each species was a composite of several batches mixed on the day of collection. The curves illustrate the marked species difference in clot density and the variation between specimens to be expected. These curves represent approximately the extremes encountered among the plasmas tested.

It may be noted also that the time of onset of clotting varies. One curve of each species shows a delay of five minutes. Both of these samples were obtained in winter. The composite samples were obtained in summer and though the inception of clotting was more rapid the final densities did not reflect the same apparent seasonal effect. Among data so far accumulated the optical density has usually been greater in plasmas collected in summer than in those collected in winter. However, in figure 1 the winter sample of sheep plasma (SE) is denser. Additional tests are needed to distinguish clearly between seasonal and individual variation. It may be added that in assay work there is a tendency for both species to require less heparin in winter than in summer thus reflecting seasonal density changes. A similar observation was made by Jorpes and his coworkers (6).

Nygaard (7) noted that the plasma clotting curve is divided into four parts.

The first phase is termed the period of dissociation. It corresponds to stage 1 of the clotting reaction and is the period from mixing to the beginning of fibrin formation, a point designated *F* by him (see fig. 1). At *F* the transmission of light

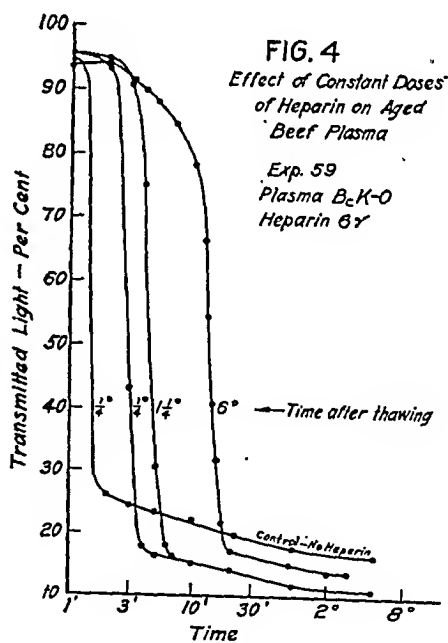
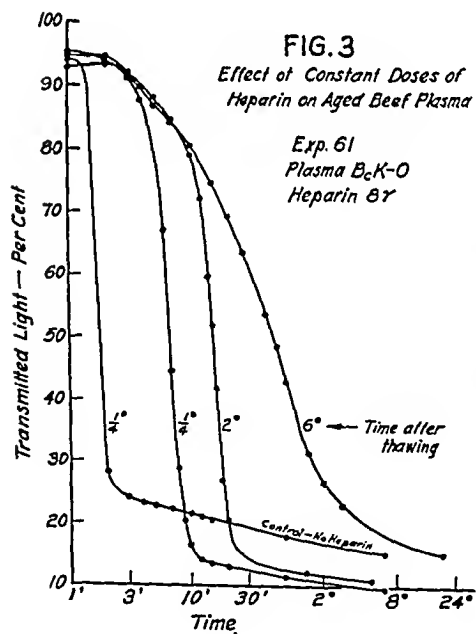
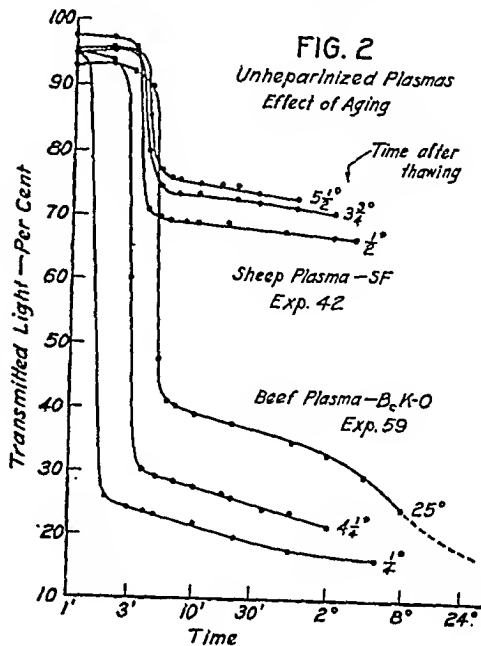
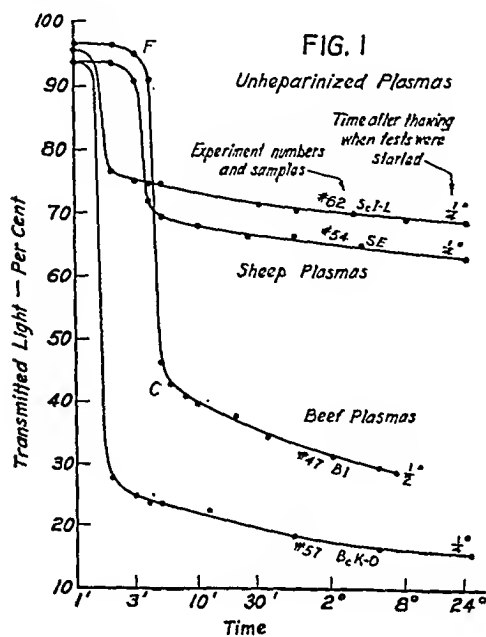


FIG. 1-4

begins to decrease rapidly. The second phase is the period of fibrin formation and is represented by the rapid descent of the curve. Fibrin formation is considered almost complete at a point *C* located where the curve begins to level off. Beyond *C* is the third phase or 'rest period'. This 'rest period' is characterized

by a further gradual decline in light transmission (or increase in density). This decline is quite apparent in the curves shown here and, in fact, proceeds much farther than in Nygaard's curves, probably attributable to the absence of clot retraction in these tests in contrast to his tests on fresh human plasma. The fourth phase is the phase of retraction, the onset designated *R* by Nygaard, and is shown by him to start within a few minutes after point *C* is reached. Retraction has never been observed in clots from frozen beef or sheep plasma, presumably because of loss of platelets. The glass surface may be a factor in preventing retraction of the colloidal aggregate (8) but retraction has not been seen in cellulose nitrate or silicone coated glass tubes.

In the third phase or 'rest period' Nygaard first considered that the further increase in density might be due to clot retraction without expression of serum. He ruled this out however by showing that further fibrin was formed during this third phase. The apparent length of this third phase was short in his experiments. If the same explanation holds for these tests, it means that coagulation is not complete for at least 8 to 24 hours. The plasmas employed here, in most instances, began to coagulate as soon or sooner than in the examples shown by Nygaard and the second phase was just as rapid. It does not seem unlikely that additional fibrin might be formed during the early part of the 'rest period', but it hardly seems credible that fibrin may still be forming for as long as 8 to 24 hours. However, the curves show a decrease in light transmission during the 'rest period' of as much as 30 per cent with or without heparin and we would then infer that 30 per cent more fibrin is formed during this period. Since the changes between the second and third phases are somewhat abrupt, this would suggest that the transformation of fibrinogen is substantially completed by this time.

It seems more plausible that some physical transformation of the fibrin already formed is taking place. Retraction—or contraction—of individual isolated fibrils may be taking place, thereby becoming shorter and thicker and with an absence of fusion to form an interlacing network would then result in no retraction of the clot as a whole. Another explanation may be that the fibers are simply imbibing fluid and swelling. Finally, a third explanation may be that the fibrils are agglutinating to form larger fibers but are not contracting. In any event a thickening of the fibrin fibers would account for the greater optical density. One of these explanations seems more likely than that fibrin formation is still going on for several hours and to the extent of 30 per cent.

Some of the terms used by Nygaard (7) do not seem appropriate. His designation of the first phase as 'dissociation' implies that the first stage of the clotting reaction is a dissociation process but, at the most, this is only the initial part of the reaction since molecules are also considered to be combining during this phase. It would seem more simplified and less committal to call the first phase the 'latent period' on these clotting curves. The second phase, that of fibrin formation, could be described as the 'coagulation period'. The third phase, or 'rest period', is certainly not characterized by rest. The term 'condensation' used by Howell (8) is more appropriate although he considered this condensation to be a part of the process of retraction. But it is manifest that condensation can occur without

clot retraction. The fourth phase, or 'retraction period', is suitably named. In this paper the first three phases will be termed the latent, coagulation and condensation periods, respectively.

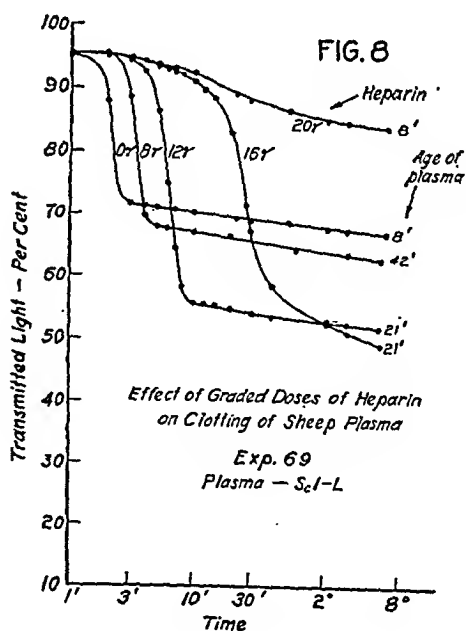
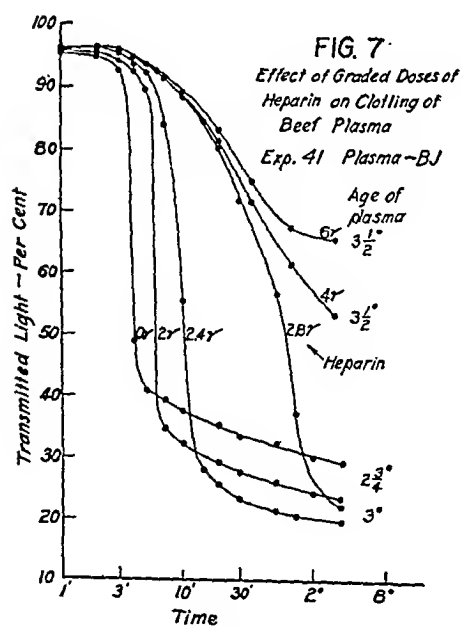
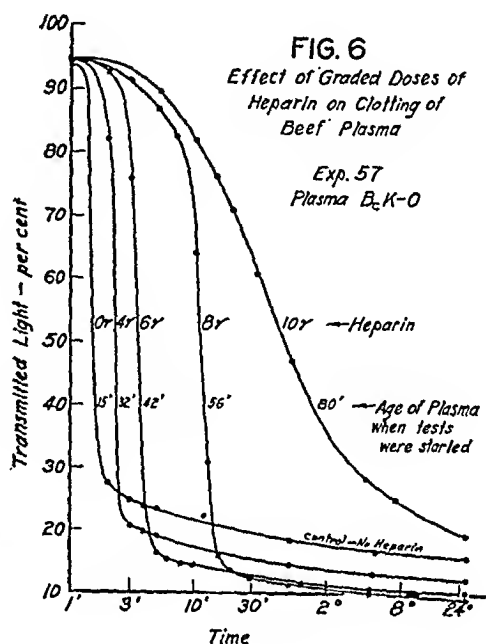
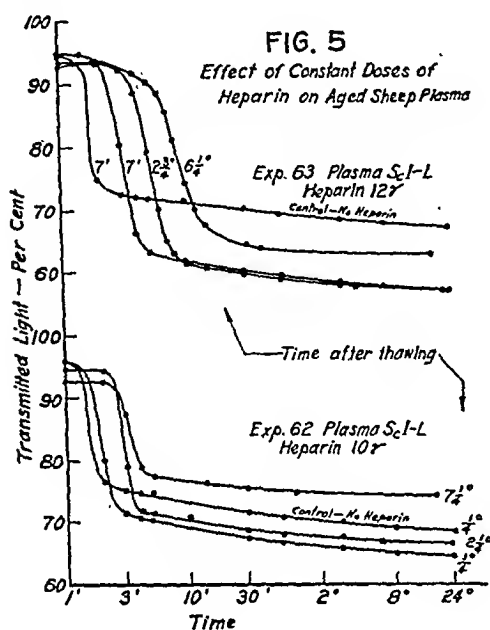


FIG. 5-8

Effect of aging of thawed plasma (fig. 2). It has long been known that plasma loses its coagulability on standing for sufficient time at room temperature. The curves in figure 2 show both a decreased final density and a delay in the onset of clotting which are evidence of this loss. The effect is more noticeable with beef

than with sheep plasma. The decreased density may be explained by a loss or denaturing of the fibrinogen. Lavergne *et al.* and Lozner *et al.* (9) have given evidence for the loss of fibrinogen in aged plasma. However, Honorato and Quick (10) do not believe that fibrinogen is lost and Honorato (11) states that fibrinogen from stored plasma clots more easily.

Effect of heparin on aged thawed plasma (fig. 3, 4 and 5). A constant concentration of heparin was used in each series of tests. Two different concentrations are shown here for both the beef and the sheep plasmas. The onset of the clotting was delayed in all instances and the delay became progressively greater as the plasma aged. The latent period is thus a summation of the aging effect and the inhibition caused by heparin (cf. fig. 2).

In all tests where heparinized and unheparinized plasma were tested simultaneously, the final density of clots formed in the presence of heparin became greater. As the plasma aged the final clot density in the presence of heparin became less but in most cases was still greater than that of the unaged control plasma. Experiment 62, figure 5, shows a case with sheep plasma where aging at last resulted in a final density less than that of the original plasma.

Effect of graded concentrations of heparin (fig. 6, 7 and 8). The tests in experiments 57 and 69, figures 6 and 8, were made as soon after thawing as possible. Since only two tests could be run simultaneously, varying lengths of time necessarily elapsed before the entire range could be covered. The aging factor has but a small influence on these results as may be concluded by comparing the curves with those of figures 2 to 5 and may be neglected in the interpretations. Nevertheless, the actual plasma ages at the time the tests were started have been indicated on the charts.

Figure 6 shows the reaction of a composite beef plasma to concentrations of heparin ranging from 4 to 10 gammas per tube (that is, per 1.5 cc. of plasma). The latent period is progressively prolonged and the maximum rate of clotting has become diminished. However, when the effect is plotted against the log of time, the steepest portions of the curves are seen to be nearly parallel with all but the highest concentration of heparin. The curves show that there is a progressive increase in the final clot density as the dose of heparin is increased until a concentration between 8 and 10 gammas is reached.

Figure 7 shows curves of a sample of beef plasma tested after it had aged about three hours. The results were the same as those in figure 6 except for the much lower range of heparin concentration. The markedly different heparin requirement is attributed to plasma variation (the plasma was collected in winter).

In figure 8 results on sheep plasma are given. The curves are quite comparable to those for beef plasma, for as the dose of heparin is increased the ultimate density of the clot increases until a critical concentration is reached where the plasma rapidly fails to clot. In the coagulation period the curves are all parallel in their steepest portions except for the one with the highest dose of heparin. The increased final density was particularly marked with 12 and 16 gammas of heparin, the proportionate increase being greater than with beef plasma and even greater than usually encountered with sheep plasma. The plasma was a summer specimen.

DISCUSSION

It is now apparent that an assay technique for heparin in which density-time clotting curves were to be established would not be practical. The chief reason for the lack of adaptability is the change in the coagulation characteristics as the plasma ages after thawing. As a means of reading the end-point in an assay by conventional techniques, the electrophotometer may be of use. At least the end-point would be purely objective and not subject to the personal errors of visual observation in the interpretation of the degree of clotting. Since the dose-effect curve is very steep (4), it is questionable whether in practical assay work the use of the electrophotometer would add significantly to the accuracy of the result. A study of this is planned however.

These studies have clearly shown the effect of heparin and aging of the plasma in modifying the character of the time-density clotting curve. Aging results in a decrease in the density of the clot and heparin within limits increases its density. Both aging and heparin tend to prolong the latent period. The rate of coagulation is decreased by heparin, but the charts show that the steepest portions of the curves are approximately parallel so the rate of fibrin formation is a constant function of the log of time up to the point where the plasma rapidly becomes incoagulable.

The explanation for effect of heparin, in increasing the density of the ultimate clot, must lie in the rate of formation of fibrin. Slow formation of the fibrin fibers results in growth to a larger size and is analogous to the precipitation and growth of crystals from solutions of crystalloidal substances. Rapid formation of particles, whether crystalloidal or colloidal in nature, results in their being smaller and more highly dispersed. Heparin controls the concentration of one of the two factors of the second stage of the clotting reaction. By keeping thrombin at a low concentration, the conversion of fibrinogen molecules is induced to proceed more slowly and to go to a degree of greater particle size and, hence, greater optical density. Howell (12) observed in the ultramicroscope that rapid clotting gave fine needles of fibrin and that slow clotting gave large needles. Wolpers and Ruska (13) in studies with the electron microscope employed heparin to slow the rate of clotting and allow the growth of larger fibers. Indirect evidence has been obtained in, as yet, incomplete experiments for attributing the density to increased particle size. Clots were subjected to high speed centrifugation (about 18,000 G), dried and weighed. In the heparin concentration range, where optical density was increasing, the clots became lighter in weight.

The question of the gradual increase in optical density of the ultimate clot during the condensation period has already been discussed.

SUMMARY

1. The changes in optical density taking place in the clotting of plasma were followed with an electrophotometer. The plasma employed had been kept frozen for several months.
2. The optical density of clotted beef plasma is about three times as great as that of clotted sheep plasma.

3. As the plasmas age after thawing there is a delay in the onset of coagulation (latent period), a slightly slower rate of coagulation and a final clot of diminished density. After the phase of rapid coagulation (coagulation period) is completed the optical density of the clots continues to increase (condensation period), probably reflecting a physical change in the fibrin.

4. Heparin in all doses prolongs the latent period.

5. With the lower concentrations of heparin the coagulation period is prolonged but the logarithm of the rate of fibrin formation is constant.

6. With the lower concentrations of heparin the final optical density of the clot becomes progressively greater as the concentration increases. With the higher concentrations of heparin the plasma becomes rapidly incoagulable.

7. The optical density continues to increase for many hours during the condensation period.

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IMPORTANCE OF PRESSURE FACTORS IN THE GENESIS OF PULMONARY EDEMA FOLLOWING VAGOTOMY¹

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Received for publication February 27, 1948

Bilateral cervical vagotomy results, in several species of animal, in pulmonary edema. Various workers, recently Lorber (1), have shown that obstruction to the airway by laryngeal paralysis and accumulation of tracheal secretions plays a large part in the pathogenesis of edema following vagotomy. They found that if a free airway is maintained lung edema does not occur. On the other hand, Farber (2) has reported the results of experiments in which guinea pigs were maintained on artificial positive pressure respiration after tracheal cannulation. He found in such animals that fatal lung edema occurred in three and one-half to four hours if bilateral cervical vagotomy had been performed, while animals with intact vagi showed only congestion after five hours. He interpreted his findings to mean that "disturbances to the vasomotor control of the pulmonary vessels . . . lead to the production of severe pulmonary edema and hemorrhage."

Since the studies of Daly (3) and others show only slight evidences of pulmonary vasomotor activity in vagal fibers it seems unlikely that the above conclusion is correct. For this and other reasons it was decided to study further the factors involved in lung edema following vagotomy in the guinea pig. As a first step the experiments of Farber were repeated. This paper is a report on the observations made.

METHODS

Adult guinea pigs of both sexes were employed. They were anesthetized with intraperitoneal nembutal, 3.0 mgm. per 100 grams. Artificial respiration by positive pressure tracheal cannula insufflation was maintained throughout. Two animals, one with cervical vagotomy and one a control, were studied simultaneously on the same respiration pump. The pressure of insufflation was varied in different experiments. Expiration was passive, at atmospheric pressure. All animals were killed by bleeding from both cut carotids. To obtain constancy of conditions for study of the lungs the lungs were not removed until bleeding had ceased with the hind legs elevated. Because no accurate chemical method of assay of pulmonary edema has been developed the state of the lungs was assessed by gross and microscopic examination. The following index was used to grade the gross lesions:

Grade 1. Slight deepening of the normal pink.

Grade 2. Moderate generalized red discoloration.

Grade 3. Deep red congestion of the lungs but no frothy fluid in the trachea or dripping from the lung surface.

¹ Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

Grade 4. Frothy serosanguinous fluid in the trachea, bloody fluid dripping from the lung surface but considerable normal lung remaining.

Grade 5. Bilateral complete involvement with extremely wet deeply hemorrhagic lungs.

TABLE 1

INTACT VAGI GROUP				VAGOTOMIZED GROUP			
Weight Sex	Duration Hours		Index of Lung Pathology	Weight Sex	Duration Hours		Index of Lung Pathology
322 M	4	KO	1	250 M	7	KO	2
260 M	5	KO	1	240 M	11	KO	3
350 M	5	KO	2	245 F	7½	KO	2
270 M	5	KO	1	366 M	9	KO	3
265 F	7½	KO	3	520 F	9½	KO	3
510 M	10	KO	1	850 M	9½	KO	2
530 F	5	K	1	510 F	9	KO	2
407 M	5	K	1	611 M	5	K	1
720 M	12	K	1	650 M	12	K	2
668 F	14	K	1	1000 M	14	K	2
794 M	25	K	3	774 M	25	K	3
Mean Index—1.5				Mean Index—2.2			

K—Killed.

O—Open chest experiment.

TABLE 2

Respirator pressure 20 mm. Hg

INTACT VAGI GROUP				VAGOTOMIZED GROUP			
Weight Sex	Duration Hours		Index of Lung Pathology	Weight Sex	Duration Hours		Index of Lung Pathology
354 M	3½	K	4	367 M	3	K	4
460 M	5	K	5	456 M	5	K	4
575 F	5	K	5	459 M	5	K	4
515 M	6	K	5	852 M	6	K	4
Mean Index—4.7				Mean Index—4			

K—Killed.

RESULTS

In Table 1 appear the results of 11 pairs of experiments in which guinea pigs were maintained on 6 mm. Hg positive pressure insufflation for periods ranging from 4 to 25 hours. It may be noted that in no instance was pulmonary edema observed. The mean index of lung pathology was slightly higher in the vagotomized than in the control group, but the former were maintained longer on artificial respiration before sacrifice in 6 of the 11 cases. The important point seems

to be that congestion was the maximum lesion seen, even in vagotomized animals, after as much as 25 hours of artificial respiration at 6 mm. Hg pressure.

On the other hand, as may be seen in table 2, when 20 mm. Hg pressure was employed in the artificial respiration an entirely different picture is found. In four pairs of experiments every animal showed massive edema within from 3 to 6 hours. In this series it may be noted that the mean index of lung pathology is lower in the vagotomized group.

It will be noted that in certain experiments in table 1 it is indicated that the chest was open. No remarkable difference was seen as a result of this maneuver. Microscopic studies were made on representative samples of lung tissues from these experiments.² The results will not be reported in detail because they merely confirm the gross observations in the case of the more severe two grades of pathology. The less severe changes seen grossly cannot be accurately distinguished in microscopic section.

DISCUSSION

These studies show that pulmonary edema does not occur either in the intact or the vagotomized guinea pig under artificial respiration, when the pressure used is 6 mm. Hg within periods of from 5 to 25 hours. Such edema does occur, however, within 5 hours in both intact and vagotomized animals when the pressure of insufflation is 20 mm. Hg. The earlier studies cited reporting such edema probably dealt with pressures in the latter range, although no mention was made of pressure conditions in the report. The present studies do not account for the reported differences between intact and vagotomized guinea pigs. A systematic difference in experimental and control animals could if it occurred, account for Farber's results. It is also possible that different results might be obtained at pressures intermediate between the two employed in the present study.

The present results seem to show quite clearly that pressure factors are critical for pulmonary edema formation in the guinea pig under artificial respiration. Furthermore, they show that under at least one pressure condition under which lung edema occurs regularly, it is not augmented by vagotomy. It seems very doubtful that vagotomy exerts any important specific effect upon the susceptibility of the lung vessels to edema or hemorrhage, although secondary effects mediated through known vagal actions on the heart, the bore of the bronchial tree, or on the reflex control of respiration, are not impossible nor even unlikely.

The observations reported at this time appear to be significant also in showing that in the guinea pig, as in the dog (4), positive pressure respiration does not prevent the development of lung edema under certain conditions. It should not, however, be concluded that under other conditions positive pressure may not impede edema formation. It is not impossible that extreme, prolonged positive pressure breathing may induce such a degree of cardiac failure as to raise left atrial and pulmonary venous pressures to very high values, thus inducing pulmonary edema. This possibility is at present being tested.

² The authors are indebted to Mr. James Weiss for preparation of the microscopic sections.

SUMMARY

1. Neither intact nor vagotomized guinea pigs developed pulmonary edema when maintained on artificial respiration at 6 mm. Hg insufflation pressure. Both intact and vagotomized animals developed massive edema within 6 hours when the insufflation pressure was raised to 20 mm. Hg.

2. Under the conditions of these experiments there is no evidence that vagotomy exerts an influence on the pulmonary vascular system favoring edema or hemorrhage.

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AN ATTEMPT TO PRODUCE PULMONARY EDEMA BY INCREASED INTRACRANIAL PRESSURE¹

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Received for publication December 10, 1947

Several reports have indicated that the central and autonomic nervous systems may be implicated in the production of some types of paroxysmal pulmonary edema (1-6). Thus Luisada (1) has claimed that in rabbits pulmonary edema of a reflex nature may be produced experimentally by inducing hypertension in the isolated head. Further, cases of paroxysmal pulmonary edema have been reported to have occurred following cranial injury (7). Our interest in the mechanism of the blood pressure changes occurring during increased intracranial pressure (8) made it appear of value to study the mechanism of the pulmonary edema which reportedly occurred under these conditions. For this purpose, we employed increased intracranial pressure which, while directly affecting the higher nervous centers, at the same time causes an increased load upon the left ventricle due to resulting elevated systemic arterial pressure.

METHODS

Dogs were anesthetized with pentobarbital sodium (25 mgm./kgm.). Arterial and intracranial pressures were measured with mercury manometers. Intracranial pressure was raised by means of a saline reservoir connected through a trephine hole in the skull, the underlying dura mater having been removed. We attempted to maintain the intracranial pressure at such a level that the resulting arterial pressure rise would be maximal, without producing acute failure of the left ventricle. Under these circumstances the factor limiting survival of the animal was the failure of respiration which always occurred before dynamic impairment of cardiac function was detected. Because of this tendency of increased intracranial pressure to cause respiratory arrest, the intracranial pressure was applied intermittently and its level was varied with the condition of the animal. Respiration was spontaneous (except as noted below), positive blast artificial respiration being avoided as an unfavorable factor for the production of pulmonary edema.

RESULTS

In two of our early experiments in which increased intracranial pressures of from 20 to 138 mm. of Hg had been maintained intermittently for periods totalling about 71 minutes, the lungs were full of a copious, thin, white, frothy fluid present in the air passages as far down as the dissecting scissors could reach

¹ Aided by the A. D. Nast Fund for Cardiovascular Research. The Department is supported in part by the Michael Reese Research Foundation.

and expressible from the cut surface of the lung. The picture grossly was that of massive pulmonary edema. However, we noted that after application of the increased intracranial pressure, many of the animals tended to produce considerable amounts of saliva, and the possibility that this saliva might be a contributing factor in the production of this picture had to be excluded. Accordingly, the experiments were repeated in nine dogs in which tracheotomy was performed so as to prevent saliva from passing down the trachea into the lungs. Some of the animals were maintained during the experiment with the head slightly dependent. Two of the animals in this group had the chest opened and received positive blast respiration which permitted the application of intracranial pressures up to levels (over 300 mm. Hg) above that which the arterial pressure could attain. One other animal received positive blast respiration with the chest closed. The other six animals breathed naturally. In none of these animals was there any evidence of pulmonary edema.

Microscopic examination of the lungs of the two original dogs without tracheal cannulae, which had showed fluid in the air passages, revealed bronchopneumonia in one dog and anthracosis in the other. No true pulmonary edema was present in either case.

DISCUSSION

Short (9) has already pointed out that the aspiration of stomach contents and the accumulation of tracheal and bronchial secretions leading to slow asphyxia may result in the development of pulmonary edema. It is apparent that the increased intracranial pressure as produced in our experiments did not produce pulmonary edema. However, our experience serves to show that a picture grossly resembling pulmonary edema may be produced by the aspiration of saliva. Our experience serves to question whether previous attempts to produce pulmonary edema by increasing intracranial pressure were successful or were due to artefact resulting from the aspiration of saliva.

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RETENTION OF SEX FUNCTIONS AFTER ISOLATION OF THE PARS ANTERIOR BY EXTIRPATION OF THE HYPOPHYSIAL STALK¹

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Received for publication February 24, 1948

A long-term cytological study of the canine hypophysis is being made with special attention directed to the tissue remaining after graded hypophysectomy procedures (1, 2). It is suspected that such an approach, when correlated with the careful assaying of the functional deficits produced in the animals, will yield information relative to the association of function with specific anatomical elements that perhaps has not hitherto been attained.

The present report summarizes the findings with respect to sex functions in a group of nine dogs in which the hypophyseal stalk tissue was surgically removed with a sparing of varying amounts of the distal portion of the pars anterior as illustrated in the diagram and photograph shown in figure 1.

MATERIALS AND METHODS

Animals. There were one male and eight female mongrel dogs of medium size used in this series. At the time of operation five animals were sexually mature, while the others were 'senior' pups. The animals were allowed to exist for periods of time ranging from 8 to 19 months.

Operative procedure. After the right lateral aspects of the hypophysis, the stalk, and the hypothalamus were exposed by the subtemporal approach, as previously described (1), the stalk tissue was removed in one of the following two ways.

In the first part of the series the stalk was cut distally with one snip of the scissors in such a manner that an appreciable rim of pars anterior tissue remained attached to the stalk tissue (see line 2, figure 1A). A small metal disc was then placed against the cut surface of the isolated hypophyseal tissue to protect it from the heat of the cauterization probe. The stalk tissue which remained attached to the hypothalamus was then cauterized.

This method was soon discarded because of 1) the uncertainty of devitalizing all the stalk tissue and 2) the frequent encroachment upon the tuberal portion of the hypothalamus. Instead, as an alternative procedure, the stalk was cut with the scissors as close to the hypothalamus as possible (see line 1, figure 1A), and a second cut was made through the proximal portion of the pars anterior (see line 2, figure 1A). The tissue freed by the two incisions was removed as a surgical specimen and stored in formalin for reference. This procedure prevented encroachment upon the hypothalamus, as well as leaving no more than a remnant of the proximal stalk attached to it.

¹ Aided by a grant from the John and Mary R. Markle Foundation.

Tissue. At autopsy the brain was removed from the floor of the skull and sella turcica in such a manner that the meninges, connective tissue, and hypophysial fragments remained in situ on its under surface.

Following fixation of the hypophysis-hypothalamic block in Zenkerformol and a subsequent period in 10 per cent formalin, the tissues were sectioned at 6 micra. An interrupted series (one section from each 20) was stained by the Mallory azan method. Another series was subjected to the copper chrome hematoxylin of Rasmussen (3). The latter stain is considered as specific for the acidophilic cells of the anterior lobe. Hansen's chromalum hematoxylin, as mentioned by Dawson (4), was used as a selective stain for the basophilic cells, but was not used for the whole series, since it was considered to have no distinct advantage over the Mallory azan method.

RESULTS

Accomplishment of operative objective. The hypophysial stalk tissue, with the exception of small fragments of proximal tuberalis and infundibulum which remained attached to the hypothalamus, was removed in all instances. This desired effect was accomplished at the expense of considerable encroachment upon the proximal pars anterior tissue and resulted in varying amounts of the distal pars anterior remaining viable. There was slight infringement upon the hypothalamus in two of the dogs, while in the others microscopic inspection revealed the hypothalamus to be undisturbed. A photomicrograph of a section taken from the series on *dog 1* is shown in figure 1B.

Retention of sex function. Completely normal sex functions were retained in *dogs 1* (female) and *2* (male), as shown by the female's whelping and rearing a litter of five puppies which were sired by the male. Sex functions were also retained in *dogs 3, 4, 5, and 6*, as judged by the fact that autopsy revealed genital tracts of normal size and serial sections of the ovaries demonstrated that the numbers and structure of the follicles were within normal limits. Regressing corpora lutea were observed in these sections in *dogs 3, 4, 5, and 6*.

Dog 1 was sexually mature at the time of operation. The first postoperative estrus period occurred in the fifth month during which she copulated with a normal male several times, and nine weeks later whelped one pup. Estrus was again manifested six months later, but she was not mated. In the third estrus period, which occurred six months later, she was mated with *dog 2* and whelped a litter of five pups.

Approximately the distal half of the pars anterior was found to have remained viable when the animal was terminated 19 months after the operation. This is shown in the photograph in figure 1B. Histologically the sections showed a normal complement of acidophiles, basophiles and chromophobe cells. The chromophilic cells were well granulated, and the arrangement of the cells in the structure of the cords did not deviate markedly from the normal. These features are illustrated in the photomicrograph shown in figure 2A.

Dog 2 was judged to be approximately three-fourths grown and was sexually immature at the time of operation. Subsequently he matured, without any

suspicion of delay, and exhibited a *persistent hyperactive sex drive*. At autopsy 16 months after operation, approximately the distal half of the pars anterior

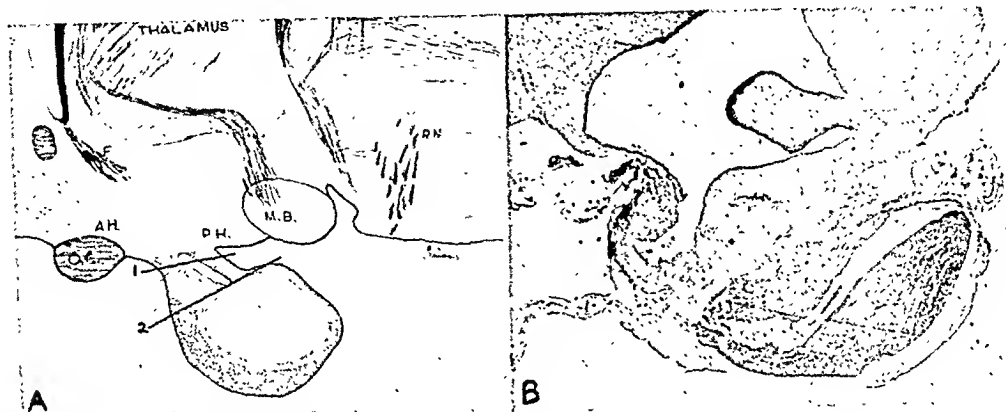


FIG. 1A. DIAGRAMMATIC REPRESENTATION OF A SAGITTAL SECTION through the hypophysis and hypothalamus to illustrate the levels of section and the amount of tissue removed as a surgical specimen.

FIG. 1B. LOW POWER PHOTOMICROGRAPH of a section of the hypophysis and hypothalamus taken from the series on *dog 1*. Optic chiasma is located to upper left of figure. Note that a fibrous layer is interposed between the infundibulum that remains and the distal hypophyseal tissue. The cleft which separates the pars anterior from the pars intermedia and the remnant of the pars nervosa is clearly evident. Note particularly that no pars tuberalis tissue remains.

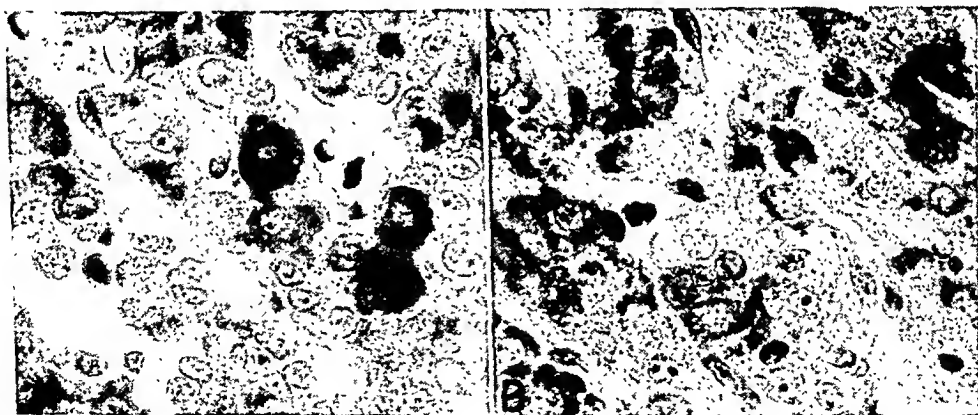


FIG. 2A. HIGH POWER PHOTOMICROGRAPH of pars anterior of *dog 1* to illustrate presence of acidophiles (dark cells); basophiles (dark gray cells); and chromophobes (lighter and smaller cells). Heidenhain azan method of staining.

FIG. 2B. PHOTOMICROGRAPH AT SAME MAGNIFICATION as 2A taken from a portion of the pars anterior remnant of *dog 3*. A decrease in the granules in both the acidophiles and basophiles is noted. Heidenhain azan method of staining.

remained intact and acidophiles, basophiles and chromophobe cells were well represented in the glandular tissue. The only deviation from a normal appearance was that the arrangement of the cells tended more to a diffuse pattern with slightly enlarged sinusoids intervening between the cords of cells.

Dog 3 was an immature female which developed to sexual maturity post-operatively; however this development was definitely delayed (see fig. 3A). Necropsy performed 16 months after operation revealed that the amount of pars anterior tissue remaining constituted about one sixth of the original total volume. Although some fully granulated cells were visible, a majority of the basophiles were found to be partially or totally degranulated.² A slight reduction in the number of acidophiles had occurred.

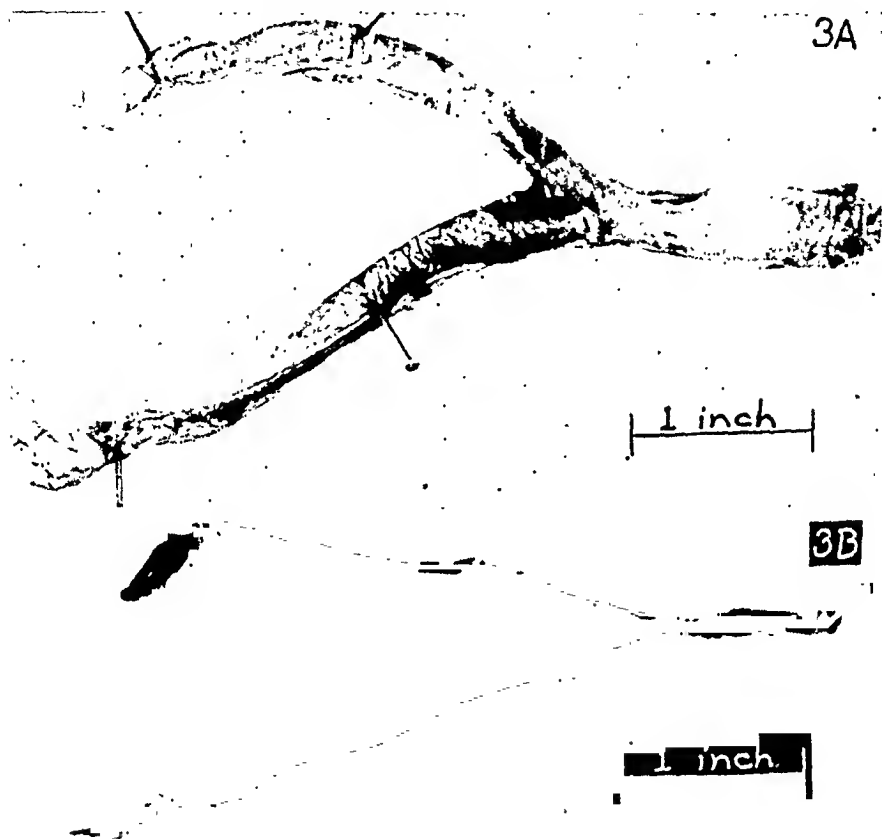


FIG. 3A. PHOTOGRAPH OF THE OVARIES AND GENITAL TRACT of *dog 3* which demonstrates complete development subsequent to the stalk removal procedure.

FIG. 3B. PHOTOGRAPH OF OVARIES AND GENITAL TRACT of *dog 8* which demonstrates the complete failure in development subsequent to the stalk removal procedure.

Gross inspection of the anterior lobe tissue in *dogs 4* and *5* showed that practically the entire pars anterior had persisted following the stalk removal procedure. The postoperative periods for these two dogs were 9 and 8 months, respectively. These tissues gave histological evidence of a slight reduction in the numbers of basophile cells when compared to normal anterior lobe tissue. The acidophiles

² The appearance of the basophiles described above may be correlated, in part, with the findings of R. Gregory and G. A. Drager, *Federation Proc.* 6: 116, 1947, in which they reported that the basophiles increased in size and became degranulated following total pancreatectomy. This dog survived total pancreatectomy four weeks.

were numerous and well granulated. Chromophobic cells did not appear to deviate from those seen in normal tissue.

The female dog 6 was retained for 10 months following the hypophyseal stalk removal procedure and at the time of termination showed that approximately one third of the normal amount of anterior lobe tissue had remained distally. Its microscopic appearance was such that the cell population did not vary noticeably from similar fields seen in an intact gland. Basophiles, filled with granules, were common in all fields and acidophilic cells were numerous and well granulated. No change could be noted in the number or character of the chromophobe cells.

Failure of development of or regression of sex organs. Of the remaining three females, dog 7 was sexually mature at operation, and necropsy eight months later showed regression of all sex organs. The other two, dogs 8 and 9, were sexually immature at the time of the hypophysectomy procedure, and retained infantile reproductive systems throughout the postoperative period of 10 months (see fig. 3B). The quantity of anterior lobe tissue viable at autopsy and later verified by microscopic observation revealed an almost total absence of granulated basophiles as well as a marked reduction in the partially granular and agranular stages of this cell type. Although fully developed acidophiles were present, there was a definite reduction in their number. Chromophobe cells predominated in all areas of the viable gland tissue, and their appearance was compatible with those seen in an intact gland.

DISCUSSION

The relation of the size of the isolated pars anterior remnant to the retention of sex functions. It is a common, and correct, practice to relate basal, reserve and remnantal endocrine functions with 1) the amount of a particular gland tissue which can be removed experimentally without producing a demonstrable deficit and 2) the over-all size of a tissue remnant which is able to prevent an extirpation deficit from being total. All of the endocrine glands that have been examined according to these criteria have been found to have tremendous reserves in terms of tissue mass. It also has been demonstrated that the smallest anatomical remnant must not be ignored when a total deficit is being assayed. The work of Smith on rats (5) gives ample evidence that these criteria obtain between gonadotropic function and the pars anterior tissue of the adenohypophysis.

It nevertheless cannot be assumed that the mere presence of a large viable tissue remnant ensures continuance of function. That a large pars anterior tissue mass may be viable while at the same time be non-functional, with respect to its control over sex functions, is well-illustrated in dogs 7 and 8 of this series. Dog 7 failed to develop sexually in spite of the fact that it had about as much recognizable viable pars anterior tissue as dog 2 and more than did dog 3. In other words, in terms of the estimation of relative size, as judged by inspection of representative serial sections stained by a non-differential stain, such as cresyl violet, one would have expected the same functional results for both dogs 2 and 7.

In the presence of a deficit, it is accordingly relatively futile to speak in terms of weight or estimated size of a viable remnant of pars anterior tissue.

Relation of the specific cytology of the isolated pars anterior tissue to the retention of sex functions. The cytology of the mammalian pituitary has been subjected to critical analyses, associated with indirect functional studies, by numerous investigators in the attempt to correlate a specific function to specific anatomical cells. As a result, gonadotropic hormone production has long been believed to be a specific function of the basophiles found in the pars anterior component of the adenohypophysis. In the present studies, the cytology of the viable isolated pars anterior tissue did not deviate markedly from the normal in those animals in which sex functions were retained. In those in which sex deficits were in evidence, the cytology of the basophiles deviated considerably from the normal. Accordingly, our results are entirely compatible with the accepted view that the basophiles are the cells which elaborate the gonadotropic principles (Severinghaus, 6; Heinbecker and Rolf, 7; Dawson, 8).

Independence of the pars anterior from any possible direct hypothalamic influence. It is apparent from this series of animals that normal sex functions may be retained in the dog following isolation of a distal portion of the pars anterior from any possible direct hypothalamic influence. These findings are in accord with those presented by Keller and Hamilton in dogs (9), Brooks and Lambert in male rabbits (10), Dempsey in guinea pigs (11) and Dempsey and Uotila in rats (12). Our present experiments, other than being made on dogs rather than on rodents, are distinctive only in extending the foregoing observations pertaining to stalk section to those following complete isolation of a *distal portion* of the pars anterior. In this respect they are somewhat equivalent to the experiments of Gncep in rats (13). It of necessity follows that interference in sex function following stalk section procedures or by hypothalamic lesions must be due to a factor or factors other than to the interruption of an essential direct hypothalamic innervation of the pars anterior.

SUMMARY

The distal portion of the pars anterior was isolated by the surgical procedure of removing the hypophysial stalk tissue and the proximal portion of the pars anterior in nine dogs.

Sexual functions remained completely intact in two dogs as evidenced by their ability to reproduce. When judged by gross and histological studies, the ovaries and genital tracts remained normal in four others of the group. In the three remaining dogs the ovaries and genital tracts, as revealed by histological studies, were markedly atrophied.

There was no correlation between retention of sex functions and the size of the isolated mass of pars anterior tissue remaining. Two of the dogs, in which there was obvious sex regression, had as much and in instances more viable pars anterior tissue remaining than did the dogs which retained sex functions.

There was a definite correlation between retention of sex functions and the cytology of the isolated pars anterior remnant. In those instances where func-

tional deficits were demonstrated, both the basophiles and acidophiles were reduced in numbers and the basophiles which were present did not stain normally.

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EFFECT OF ADRENALECTOMY UPON THE TOLERANCE OF THE EVISCERATED RAT FOR INTRAVENOUSLY ADMINISTERED GLUCOSE

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Received for publication January 27, 1948

In these experiments eviscerated and adrenalectomized-eviscerated rats were given continuous intravenous infusions of glucose with and without insulin for periods of 4 and 24 hours. At the end of 4 hours the average values for blood glucose were significantly lower in the adrenalectomized than in the non-adrenalectomized series at each glucose load and insulin dose. Within the 24-hour period the adrenalectomized animals removed glucose from the blood more rapidly than nonadrenalectomized animals when little or no insulin was given and the glucose loads remained low. When the comparisons were made at higher levels of insulin dosage and of glucose load the order of tolerance was reversed in that the adrenalectomized animals had higher average terminal values for blood glucose than the non-adrenalectomized rats.

METHODS

Male rats of the Sprague-Dawley strain were fed Friskies Dog Cubes. At a weight of 185 to 205 grams, the inferior vena cava was ligated between the liver and kidneys in order to cause the development of a collateral circulation. Asepsis was preserved in this operation. When the animals reached a weight of 250 (± 2) grams they were anesthetized (intraperitoneal injection of 18 mgm. of cyclopentenyl-allyl-barbituric acid sodium) and eviscerated by the procedure of Ingle and Griffith (1). The adrenal glands were ligated and removed in the same operation. Hemostasis was attained by applying a gelatin sponge (Gel-foam, Upjohn) saturated with a solution of thrombin to the stumps of the oesophagus, colon, ligated vessels and between the muscle and the skin when the incisions were closed.

Intravenous injections of solutions containing 0.9 per cent sodium chloride and varying concentrations of glucose (C.P. Dextrose, Merck) with and without crystalline zinc insulin (Lilly) were made by two continuous injection machines which delivered fluid from each syringe at the rate of 20 cc. in 24 hours. The glucose load is expressed as milligrams of glucose per 100 grams of rat per hour (mgm/100/h). Syringes of the Luer-Lok type (Becton-Dickinson) were selected to deliver 20 cc. with a stroke of 65 mm. Six syringes were operated by each machine. The machines were powered by synchronous motors, and the reduction of motion was achieved by a precision-built system of gears which exactly controlled the rate of injection.

The infusions were made into the saphenous vein of the right hind leg and were started within five minutes following the removal of the liver. The animals

were secured in a supine position on an animal board and were enclosed in a cabinet with the temperature constant at $26.5 (\pm 0.5)$ degrees C. The temperature of the room was maintained at 74 to 78 degrees F. and the humidity at 30 to 35 per cent of saturation. The more rigid control of temperature has decreased the variability of results and has slightly increased the tolerance for glucose over average values reported earlier (2). As the temperature rises, the insulin sensitivity of the animal is increased. A change of as little as one degree C. significantly changes the glucose requirement of the insulin-treated eviscerate rat. A complete control of temperature and humidity would be desirable.

The analyses of blood glucose were made by the method of Miller and Van Slyke (4). In addition to glucose, this method measures small amounts of non-fermentable reducing substances which accumulate in the blood of eviscerate rats. Samples of blood were taken from the jugular vein at the end of 4 hours of

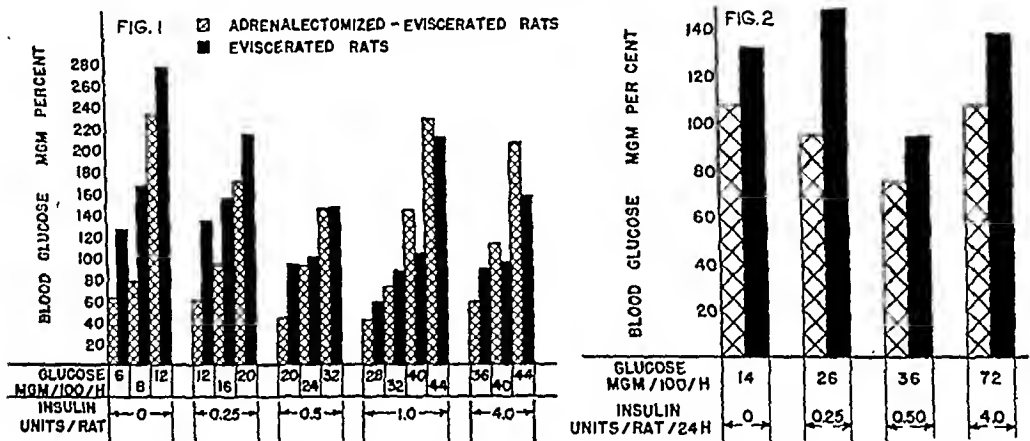


FIG. 1. AVERAGE VALUES FOR BLOOD GLUCOSE at the end of 4 hours of continuous intravenous infusion. Eviscerated rats (■) and adrenalectomized-eviscerated (▨) rats.

FIG. 2. AVERAGE VALUES FOR BLOOD GLUCOSE at the end of 24 hours of continuous intravenous infusion.

infusion in experiment 1 and at the end of 24 hours of infusion in experiment 2. The urine was tested for glucose at the end of each experiment but was negative (less than 40 mgm. reducing substances) for each animal.

EXPERIMENTS AND RESULTS

In experiment 1 (fig. 1) 4 groups of rats having 20 pairs of rats in each group were infused with glucose for a period of 4 hours. One rat of each pair was adrenalectomized. The first group was given a glucose load of 14/100/h. without insulin; the second group was given a glucose load of 26/100/h. with insulin at the rate of 0.25 units per 24 hours per rat; the third group was given a glucose load of 36/100/h. with insulin at the rate of 0.5 units per 24 hours per rat; and the fourth group was given a glucose load of 72/100/h. with insulin at the rate of 4 units per 24 hours per rat. In each group the average level of blood glucose at the end of 4 hours was significantly lower in the adrenalectomized as compared to the non-adrenalectomized animals.

In experiment 2 (fig. 2) the tolerance for glucose was determined over a 24-hour period. Twelve pairs of rats were tested in each group. Three groups of rats without insulin were studied at glucose loads of 6, 8 and 12/100/h.; at an insulin dose of 0.25 units 3 groups of rats were tested at 12, 16 and 20/100/h.; at an insulin dose of 0.5 units 3 groups of rats were tested at 20, 24 and 32/100/h.; at an insulin dose of 1.0 units 4 groups of rats were tested at 28, 32, 40 and 44/100/h.; and at an insulin dose of 4 units 3 groups of rats were tested at 36, 40 and 44/100/h.

In eviscerated rats without insulin and at a dose of 0.25 units of insulin the adrenalectomized rat removed glucose from its blood at a more rapid rate than did the non-adrenalectomized rats as indicated by the significantly lower terminal averages for blood glucose. At higher insulin dosages the average values for blood glucose in the adrenalectomized rats remained lower when the glucose loads were relatively low for that insulin dose. At glucose loads of 40 and 44/100/h. the adrenalectomized animals showed higher average rises in blood glucose than the non-adrenalectomized animals.

DISCUSSION

This study provides additional evidence that the adrenal cortical hormones affect carbohydrate metabolism in the absence of the liver. Russell (6) and Roberts (5) have each observed that adrenalectomy accelerates the rate at which glucose disappears from the blood of the eviscerated rat. In an earlier study from this laboratory (2) it was shown that within a 24-hour period the administration of large amounts of adrenal cortex extract definitely depressed the tolerance for glucose in those rats which received insulin. In the absence of insulin the effect of adrenal cortex extract upon glucose tolerance was either absent or questionable. For this reason it was anticipated that the effect of adrenalectomy would be the greatest in those animals which were treated with insulin. The contrary was true for the greatest effect of adrenalectomy upon glucose tolerance occurred in animals which received little or no insulin. The following rationalization of these apparently paradoxical results is offered without the assumption that it is the only possible explanation.

The utilization (storage, oxidation and conversion) of glucose by the eviscerated rat without insulin over a 24-hour period is very small, about 4 mgm. of glucose per 100 grams of rat per hour. Possibly this low level of glucose utilization is physiologically basal and cannot be inhibited further by agents such as an excess of the adrenal cortical hormones. Glucose utilization could, however, be shifted in the upward direction by conditions such as adrenal cortical insufficiency which favor increased glucose utilization. In the presence of optimal amounts of insulin the inactive eviscerated rat can utilize about 40 mgm. of glucose per 100 grams of rat per hour. Possibly this rate of utilization should be regarded as a physiological ceiling for the inactive eviscerated rat which cannot be increased by adrenal cortical insufficiency. Factors which favor inhibition of glucose utilization such as the adrenal cortical hormones could readily manifest their effects upon the ceiling of utilization but not upon basal

utilization. To continue speculation, why is the utilization of glucose apparently less in the adrenalectomized-eviscerated rat when the glucose load is high? This may be the result of the comparative inefficiency with which the adrenalectomized animal carries out any metabolic process when it is pushed to its physiological limit. The adrenalectomized-eviscerated animal is approaching death more rapidly than a similar animal which is supplied with the cortical hormones. It is not necessary to assume the existence of a physiological ceiling for the utilization of glucose. It can be postulated that the secondary inefficiencies of the adrenally insufficient animal completely mask the more primary effect of adrenal cortical insufficiency at high glucose loads. An analogical situation obtains in respect to the ability of the adrenalectomized dog to excrete sodium and chloride. At the usual levels of sodium chloride intake the adrenalectomized animal excretes these ions more rapidly than normal. However, when the intake of sodium chloride is increased to the limit of tolerance the ability of the adrenalectomized animal to excrete the excess of these ions is less than normal (3).

The above rationalization of the results from the 24-hour tests is weakened by the differences in results of the 4-hour and 24-hour tests. The administration of large amounts of adrenal cortex extract to the eviscerated rat (2) did not have any significant effect within a period of 4 hours whereas the effects of adrenalectomy were evident in animals at each glucose load either with or without insulin. Possibly within a period of 4 hours the major effect of adrenalectomy is to release the inhibitory effect of the cortical hormones upon glucose utilization prior to the onset of the secondary inefficiency of the adrenalectomized-eviscerated rat which develops at a later time as the animal approaches death.

The results of this study give some support to the conclusion that the effect of the adrenal cortex upon carbohydrate utilization is not dependent upon the presence of insulin. Although the pancreas has been completely removed from the eviscerated rat, it is still possible, however, that some insulin remains in the blood and tissues. Our data do not exclude the possibility that in the eviscerated rat the effect of cortical hormone deficiency and excess upon the tolerance for glucose is based upon the change in the rate of gluconeogenesis in the kidney and other extra-hepatic tissues. We recognize the possibility that adrenal medullary insufficiency may have been a factor in causing the differences between the adrenalectomized and nonadrenalectomized rat.

SUMMARY

Male rats (185-205 gram) of the Sprague-Dawley strain were caused to develop a collateral circulation by ligation of the inferior vena cava. At a weight of 250 ± 2 grams the animals were anesthetized (cyclopal), and all of the intra-abdominal organs were removed except the kidneys. The adrenal glands were removed in one animal of each pair in the same operation. Infusions into the saphenous vein were made by continuous injection machines which delivered fluid at the rate of 20 cc. in 24 hours per rat. The level of blood glucose at the end of the injection period was the index of glucose tolerance.

In experiment 1, 4 groups of rats having 20 pairs of rats in each group were

infused with glucose with and without insulin for a period of 4 hours. One rat of each pair was adrenalectomized. At each glucose load with and without insulin the average level of blood glucose at the end of 4 hours was significantly lower in the adrenalectomized than in the non-adrenalectomized animals.

In experiment 2 the tolerance for glucose was determined over a 24-hour period. Sixteen groups of rats having 12 pairs of animals in each group were infused with different loads of glucose with and without insulin. Within the 24-hour period the adrenalectomized animals removed glucose from the blood more rapidly than the non-adrenalectomized animals when little or no insulin was given and the glucose loads remained low. At the higher levels of insulin dosage and glucose loads the order of tolerance was reversed and the adrenalectomized animals had higher average terminal values for blood glucose than the non-adrenalectomized rats.

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EFFECT OF ADRENALECTOMY UPON THE URINARY EXCRETION OF GLUCOSE AND NONPROTEIN NITROGEN IN THE PARTIALLY DEPANCREATIZED, FORCE-FED RAT

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Received for publication February 2, 1948

Removal of the adrenal cortex from depancreatized animals results in amelioration of the symptoms of diabetes, whereas administration of adrenal cortex extracts and C₁₁-oxygenated cortical steroids cause exacerbation of the symptoms of diabetes. Earlier studies (1, 2) on the mechanism of the relationship between adrenal cortical function and carbohydrate metabolism showed that the reduction of glycosuria following adrenalectomy was accompanied by a decrease in urinary nitrogen, whereas the administration of diabetogenic amounts of the cortical hormones caused an increased breakdown of protein as evidenced by increased nitrogen loss. It was postulated that the principal effect of the cortical hormones upon organic metabolism was upon gluconeogenesis from protein. Subsequent studies (3-6) have indicated that the cortical hormones also affect the utilization of carbohydrate. The utilization of carbohydrate is increased by cortical insufficiency and depressed by an excess of the cortical hormones.

An appraisal of the role of cortical insufficiency in the decrease of urinary nitrogen which follows adrenalectomy in the diabetic animal should include the two following considerations: a) The diabetic animal characteristically eats larger than normal amounts of food and the untreated adrenalectomized animal eats smaller than normal amounts of food. The change in protein intake must be responsible for a part of the change in nitrogen excretion which follows adrenalectomy in the diabetic animal. b) Nitrogen loss is characteristically higher than normal in the diabetic animal and amelioration of diabetes is followed by restoration of a normal nitrogen balance. The possibility that the decrease in nitrogen loss following adrenalectomy in the depancreatized animal is secondary to improvement in glucose utilization deserves consideration.

In these experiments partially depancreatized rats were given a constant food intake by stomach tube. Following adrenalectomy there was a dissociation between the changes in urinary glucose and urinary nonprotein nitrogen. The decrease in urinary nitrogen was much too small to account for the decrease in glycosuria as being due to a decrease in gluconeogenesis from protein.

METHODS

Male rats of the Sprague-Dawley strain which were completely free from infection were used. The stock diet was Friskies Dog Cubes. At a weight of approximately 300 grams the animals were partially depancreatized by the method of Ingle and Griffith (7). After diabetes was established all of the animals were placed in metabolism cages and were fed a medium carbohydrate

diet made according to table 1. All of the animals were force-fed by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (8). During the period of adaptation to forced feeding, the amount of diet was increased gradually to prevent the development of 'food-shock.' The animals were brought to a full feeding of 26 cc. of diet per rat per day on the fifth day. The animals were kept in an air-conditioned room with temperature at 74 to 78° F. and humidity at 30 to 35 per cent of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol and added citric acid (one gram per sample) to insure the acidity of the urine for nitrogen analyses. Urine glucose was determined by the method of Benedict (9), and the determination of urinary non-protein nitrogen was by the micro-Kjeldahl procedure as follows: proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure. The

TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour (Chicago Dietetic Supply).....	120
Osborne & Mendel salt mixture.....	40
Diet yeast (Pabst).....	100
Wheat germ oil.....	10
Cod liver oil.....	10
Vitamin K (2-methyl-1,4-naphthoquinone).....	100 mgm.
Mazola oil.....	200
Casein (Labco).....	160
Starch.....	200
Dextrin.....	190
Sucrose.....	200
Water to make total of.....	2000 cc.

organic matter was oxidized by sulfuric acid and hydrogen peroxide. The ammonia was distilled off into a standard acid solution and titrated with standard base.

Following a control period all of the animals were adrenalectomized by the method of Ingle and Griffith (7). Sterile technique was used and infections were successfully avoided. The animals were given a one per cent sodium chloride solution to drink throughout the entire experiment. Beef adrenal extract (Upjohn) representing 40 grams of gland per cc. was used. It was free from alcohol.

EXPERIMENTS AND RESULTS

In experiment 1 (fig. 1) eight partially depancreatized, mildly diabetic rats were adrenalectomized following a control period of seven days. Following adrenalectomy they were maintained by drinking saline for 14 days. At this time cortical extract was administered according to the following: one cc. per day for seven days, 2 cc. per day for seven days and 4 cc. per day for seven days. At the same time, seven normal rats were studied under the same conditions of

forced feeding. The average daily value (231 mgm.) for urinary N.P.N. is shown for comparative purposes in figure 1.

Prior to adrenalectomy the diabetic rats excreted higher than normal amounts of nitrogen and an average of more than 3 grams of glucose per day. Following adrenalectomy the excretion of nitrogen was depressed for one day, but by the third day it increased above the preoperative level. This rise occurred at a time when the level of urinary glucose was falling. There was a secondary fall in urinary nitrogen below the preadrenalectomy level, but it did not fall below

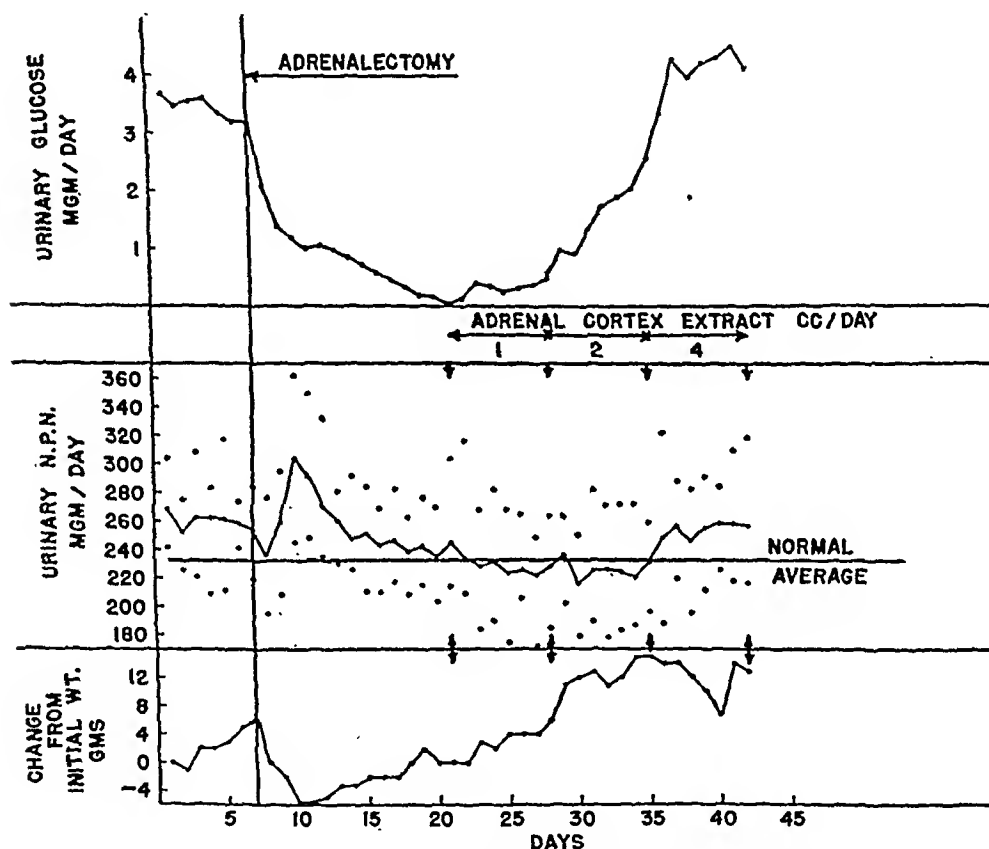


FIG. 1. PARTIALLY DEPANCREATIZED, FORCED-FED RATS. Average values for eight animals. The range of individual values is given for urinary N.P.N.

the average for normal rats during the first 14 days. The glycosuria was abolished for each rat by the end of this period. During the administration of one cc. and 2 cc. of cortical extract per day the average level of urinary nitrogen fell below the normal average although glycosuria was re-established. When the amount of cortical extract was increased to 4 cc. per day the values for both urinary nitrogen and glucose were elevated to preadrenalectomy levels and above.

In experiment 2 an attempt was made to determine the effects of adrenalectomy upon severely diabetic rats. Six extensively depancreatized rats were adrenalectomized but only three of them survived the 14-day period during which

treatment was limited to the drinking of saline. The results on the three animals which survived were in accord with the results of experiment 1. Prior to adrenalectomy these animals excreted an average of 4.5 grams of glucose and 283 mgm. of nonprotein nitrogen daily. Following adrenalectomy the glycosuria fell to an average daily value of 2.3 grams by the end of 14 days, and the corresponding value for urinary nitrogen was 252 mgm. Again, there was a decrease in urinary nitrogen immediately following adrenalectomy and a subsequent temporary rise. During treatment with one cc. and 2 cc. of cortical extract per day the glycosuria increased, while the level of urinary nitrogen decreased. During treatment with 4 cc. per day the levels of urinary nitrogen and glucose were elevated to preadrenalectomy levels. These data are not charted.

DISCUSSION

Janes, Dawson and Myers (10) found that amelioration of alloxan diabetes by adrenalectomy in rats which ate ad libitum was associated with a lowered food intake and that nonadrenalectomized, diabetic rats which were limited to the same food intake showed a similar decrease in glycosuria. The present study confirms the observations of Ingle and Thorn (5) in showing that adrenalectomy caused an amelioration of pancreatic diabetes in rats which had a normal caloric intake sustained by tube feeding. The intestinal absorption of carbohydrate in these animals was complete.

The decrease in urinary glucose following adrenalectomy (approximately 3.5 grams) cannot be accounted for in terms of decreased gluconeogenesis from protein when changes in urinary nonprotein nitrogen are used as an index of the extent of conversion. Even if it were assumed that all of the carbon of the catabolized protein was converted to carbohydrate prior to the adrenalectomy and none following adrenalectomy the change in urinary glucose was greater. There was a postadrenalectomy rise in urinary nitrogen which coincided with a period of rapid fall in the glycosuria. An additional dissociation in the direction of change in urinary glucose and nitrogen occurred during the injection of one cc. and 2 cc. daily of cortical extract when the level of urinary nitrogen decreased and glycosuria was reinduced up to average values of more than 2 grams per day.

These results are in agreement with experiments on cortical hormone overdosage in partially depancreatized (5) and normal (11) force-fed rats, in which it was shown that the glucose loss is too great to be accounted for in terms of increased protein breakdown.

None of our experiments has tested the possibility that the cortical hormones affect gluconeogenesis from fat. However, in the liverless rat glucose tolerance is increased by adrenal cortical insufficiency (12) and is depressed by cortical hormone excess (13). In our opinion the hypothesis which most nearly meets the experimental facts is the general explanation advanced by Evans (6) that the cortical hormones affect some mechanism which spares carbohydrate. Accordingly, the adrenalectomized, diabetic animal regains ability to utilize some of the carbohydrate which was formerly wasted into the urine, and cortical hormone overdosage interferes with the utilization of carbohydrate. The phase of utiliza-

tion involved may or may not be that of oxidation. The manner in which this effect of cortical insufficiency and excess relates to the role of the cortical hormones in body economy is not known.

There was some decrease in the level of urinary nitrogen following adrenalectomy but it did not fall below the average for normal animals during the 14-day postoperative period when no hormone was given. Diabetic animals characteristically excrete greater than normal amounts of nitrogen but a normal nitrogen balance is established when the glycosuria is controlled by insulin. Was the decrease in urinary nitrogen following adrenalectomy in these diabetic animals secondary to an improved utilization of glucose or does it reflect a more primary function of the cortical hormones? It has been generally considered that protein catabolism is decreased by cortical insufficiency but other studies from this laboratory (14, 15) have indicated that the force-fed, saline-treated, adrenalectomized rat is able to catabolize protein in a normal manner. Such animals do fail to mobilize their tissue proteins at a normal rate during a severe stress (15) unless they are sustained with cortical hormones.

When the administration of cortical extract was started after 14 days of saline treatment the nitrogen balance became more strongly positive. Did this represent an effect of the cortical extract? It is possible that this small change would have occurred without the change in treatment. There was a corresponding increase in body weight (fig. 1). It has been observed on several occasions in this laboratory that the force-fed, adrenalectomized rat does not gain weight quite as rapidly as the nonadrenalectomized animal. It is possible that the cortical hormone is essential for an optimal rate of anabolism although this is contrary to the generally accepted concept that the cortical hormones stimulate catabolic processes. This may only be true of cortical hormone overdosage and is not necessarily incompatible with the assumption that under some conditions physiological amounts of these hormones may support anabolism.

The amounts of beef adrenal extract required to restore the glycosuria of the adrenalectomized-depancreatized rats to its preadrenalectomy level are large when compared to doses adequate to sustain life (16). From these data (fig. 1) it can be estimated that prior to adrenalectomy the adrenal cortices of these animals secreted the activity equivalent of 3 to 4 cc. of extract per day. It is probable that the utilization of the endogenous secretion is more efficient than the intermittent injections of cortical extract. There are possible fallacies in all methods of estimating the secretory capacity of the adrenal cortices but all methods support the conclusion that very large amounts of cortical extracts are required to fully replace the functional activity of the intact adrenal glands (17).

SUMMARY

Partially depancreatized male rats of the Sprague-Dawley strain were force-fed a medium carbohydrate diet and were given a one per cent solution of sodium chloride to drink throughout the experiment. They excreted an average of 3.5 to 4.0 grams of glucose per day and the nitrogen loss was greater than normal. Following adrenalectomy there was a sharp increase in urinary nitrogen by the

third day, which coincided with a fall in the level of urinary glucose. The glycosuria disappeared and the urinary nitrogen gradually fell to normal values, but not below, during the 14 days that the animals were maintained without cortical extract. During the administration of one cc. and 2 cc. of beef adrenal extract per day the average level of urinary nitrogen fell below the normal average, although glycosuria was re-established. When the amount of cortical extract was increased to 4 cc. per day the values for both urinary nitrogen and glucose were elevated to preadrenalectomy levels and above. A decrease in gluconeogenesis from protein, as evidenced by the change in urinary nitrogen, could account for less glucose than the actual decrease of 3500 mgm. per day in urinary glucose (exp. 1). Adrenalectomy either caused a decrease in gluconeogenesis from fat or the animals regained the ability to utilize the dietary carbohydrate which they wasted by excretion prior to adrenalectomy.

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INFLUENCE OF A PREVIOUS INJECTION OF EPINEPHRINE UPON THE DIABETOGENIC EFFECT OF ALLOXAN IN RABBITS

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Received for publication May 15, 1947

During the course of studies on the action of alloxan on rabbits, an attempt was made to prevent the well-known noxious action of this drug on the kidney by interrupting the renal circulation for about six minutes at the time of the alloxan injection. It was found that rabbits thus treated either did not become diabetic or developed an extremely mild diabetes. One explanation for the protective effect observed is that our operative procedure resulted in an epinephrine discharge. Kass and Weisbren (1) have recently described a similar protective effect in rats by injection of epinephrine previous to the injection of alloxan. This prompted us to investigate the influence of a previous injection of epinephrine upon the diabetogenic effect of alloxan on rabbits.

METHODS

Twenty-two rabbits weighing 950 to 1925 grams (average 1400 grams) were given intravenous injections of commonly used diabetogenic doses of alloxan (170 to 204 mgm. per kilo of body weight) preceded by injections of epinephrine. Those that survived longer than 24 hours were kept in metabolic cages and their urine was collected daily and tested for dextrose (Benedict). Blood sugar and occasionally blood urea were measured in blood samples from the marginal ear vein by means of the Leitz electrophotometer (slightly modified Folin and Wu and Karr methods). Blood sugar figures given below are mgm. of dextrose per 100 cc. of blood. Dextrose tolerance tests were performed after 16 to 20 hours fast by measuring blood sugar level before, and two hours after, the injection of 4 cc. of 50 per cent dextrose solution per kilo of body weight. When the fasting blood sugar level was lower than 150 and the two-hour figure was not higher than the first one, the result was considered as showing the absence of a diabetic disturbance; when the two-hour figure was higher than 150, and also distinctly higher than the fasting one, the result was considered as showing the presence of a diabetic disturbance; other results would be considered doubtful. Further details concerning the experimental conditions are given in the tables.

RESULTS AND DISCUSSION

The results of our experiments are given in tables 1, 2 and 3. Out of a total number of 22 animals treated with epinephrine-alloxan, as detailed above, 11 survived longer than 24 hours (table 1). At a time when a large proportion of

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TABLE 1. DATA CONCERNING 11 RABBITS (OUT OF A SERIES OF 22) THAT SURVIVED NOT LESS THAN 24 HOURS AFTER AN INTRAVENOUS INJECTION OF A CURRENT DIABETOGENIC DOSE OF ALLOXAN PRECEDED BY AN INTRAMUSCULAR INJECTION OF A DOSE OF 200 MICROGRAMS PER KGM. OF EPINEPHRINE

RABBIT NO.	WEIGHT	AL-LOXAN PER KGM.	INTER-VAL BETWEEN INJECTIONS	LENGTH OF FASTING PRIOR TO INJECTIONS	TIME OF ONSET OF HYPOLYCEMIA AFTER AL-LOXAN INJECTION ¹	GLYCO-SURIA LATER THAN 24 HOURS AFTER AL-LOXAN INJECTION	FASTING BLOOD SUGAR			SURVIVAL PERIOD	CAUSE OF DEATH ²
							24 hours after alloxan injection	48 hours after alloxan injection	Highest value found after 48 hours		
	grams	mgm.	mins.	hours	hours		mgm/100 cc.	mgm/100 cc.	mgm/100 cc.		
1	1750	185	60	24	2	—	77		126 (12th) day	7 months	Killed
4	1650	200	60	24	4		90			less than 48 hours	Liver damage
5	1750	180	60	none	8	—	117	100	100 (8th) day	12 months	Killed
6	1240	200	60	24	3	—	37	160	97 (4th) day	4 days	Liver damage
8	1150	200	60	24	2		62			less than 48 hours	Uremia
9	1350	170	60	24	2	+		136	322 (4th) day	4 days	Killed
13	1270	200	20	none	7		408			less than 48 hours	Uremia
14	1320	200	20	none	5½	—	410			8 days	Liver damage
19	1635	180	60	16	5	—	124		124 (6th) day	12 months	Killed
20	1225	180	80	16	7	—	65	50		49 hours	Liver damage
21	1650	180	80	none		—	171	132	109 (5th) day	12 months	Killed

¹ Rabbit 21 did not show hypoglycemia at any time; it was given dextrose injections, beginning five hours after alloxan. Dextrose injections, beginning 2 to 3 hours after alloxan (before hypoglycemia was ascertained), were given to rabbits 13, 14, 19 and 20. Dextrose injections, beginning after hypoglycemia was ascertained, were given to rabbits 1, 4, 5, 6, 8 and 9.

² For rabbits 4, 6, 14 and 20 liver damage is given as the cause of death because in all these animals extremely severe liver necrosis was found by histological examination. Rabbits 8 and 13 were not subject to postmortem examination; uremia is given as the cause of their death because the 24-hour blood urea was 103 mgm.% for no. 8 and 140 mgm.% for no. 13; liver damage, as contributory factor, is of course probable.

commonly alloxanized rabbits already show an unmistakable diabetic hyperglycemia, namely 24 hours after the alloxan injection (2, 3, 4), in only 3 of our 11

surviving animals was blood sugar higher than 125. Eight of our rabbits survived 48 hours or longer. At a time when nearly all commonly alloxanized rabbits already show unmistakable diabetic hyperglycemia, namely 48 hours after the alloxan injection (2, 3, 4), blood sugar was measured in five animals and in none was it found to be higher than 160; *rabbit no. 9*, with a 48-hour blood sugar figure of only 136, showed glycosuria on the third and a blood sugar of 322 on the fourth day. None of the other six animals surviving longer than 48 hours (*nos. 1, 5, 6, 14, 19, and 21*) ever showed either glycosuria or fasting hyperglycemia later than 48 hours after the alloxan injection.

Sugar tolerance tests (table 2) were performed on five of our rabbits that survived longer than four days (see table 1), all of which failed to show glycosuria. A latent diabetic disturbance was found to exist in two (*nos. 14 and 19*) and total absence of any diabetic disturbance was observed in three (*nos. 1, 5 and 21*).

TABLE 2. DEXTROSE TOLERANCE TESTS IN FIVE RABBITS THAT SURVIVED LONGER THAN FOUR DAYS AFTER THE EPINEPHRINE ALLOXAN TREATMENT AND SHOWED NO GLYCOSURIA LATER THAN 24 HOURS AFTER THE ALLOXAN INJECTION

RABBIT NO.	BLOOD SUGAR 24 HOURS AFTER ALLOXAN INJECTION	HIGHEST FAST- ING BLOOD SUGAR LATER THAN 24 HOURS	SUGAR TOLERANCE TEST			RESULTS
			Day after epinephrine- alloxan inject- ions	Blood sugar		
				Fasting	2 hours	
	mgm/100 cc.	mgm/100 cc.		mgm/100 cc.	mgm/100 cc.	
1	77		12th	126	117	No diabetes ¹
5	117	100	6th	93	93	No diabetes ¹
14	410		8th	38	155	Latent diabetes
19	124	124	6th	124	320	Latent diabetes
21	171	132	5th	109	95	No diabetes ¹

¹ A few days after the dextrose tolerance test had shown absence of any diabetic disturbance, *rabbits no. 1, 5 and 21* were again injected with alloxan, without previous epinephrine injection, and all three developed typical diabetes.

In these three rabbits alloxan was again given a few days later, without a previous epinephrine injection, and all three developed typical diabetes. This proved that the previous failure of these animals to become diabetic was not due to an individual lack of sensitivity to alloxan. The same was shown, in quite a different way (table 1), for two of these rabbits (*nos. 1 and 5*), as well as for others that failed to show any evidence of typical diabetes (*nos. 4, 6, 8, 19 and 20*), by the fact that all these animals showed alloxan hypoglycemia.

At least in two epinephrine alloxan-treated rabbits that died from alloxan hypoglycemia (*nos. 11 and 12*, table 3), the pancreatic islets, as well as the rest of the pancreas, were found to be histologically normal. From the presented facts the statement seems justified that, under the conditions of our experiments, intramuscular injection of epinephrine in doses of 200 micrograms per kgm. of body weight before the administration of commonly used diabetogenic doses of alloxan, in most cases, either very markedly hindered or totally abolished the diabetogenic effect of alloxan in rabbits.

The above results appear to be independent of sex, age or weight of the rabbits used and of fasting or nonfasting prior to the experiment.

The fact that all the rabbits that were protected by epinephrine against the diabetogenic action of alloxan, and that were not given dextrose injections early after the alloxan treatment, showed hypoglycemia suggests that alloxan was not chemically inactivated by epinephrine. Furthermore, the variability of the antidiabetic effect of epinephrine under similar experimental conditions is also more in accordance with a biological than with a simple chemical mechanism. The biological mechanism that protects the pancreas against the diabetogenic action of alloxan may well be the vasoconstriction which is known to be elicited

TABLE 3. DATA CONCERNING 11 RABBITS (OUT OF A SERIES OF 22) THAT DIED WITHIN 24 HOURS AFTER AN INTRAVENOUS INJECTION OF A CURRENT DIABETOGENIC DOSE OF ALLOXAN PRECEDED BY AN INTRAMUSCULAR INJECTION OF A DOSE OF 200 MICROGRAMS PER KGM. OF EPINEPHRINE

RABBIT NO.	WEIGHT	ALLOXAN PER KGM.	PRELIMI- NARY FAST	TIME AFTER ALLOXAN WHEN CON- VULSIONS APPEARED FIRST	TIME AFTER ALLOXAN WHEN DEXTROSE IN- JECTIONS WERE GIVEN		TIME OF DEATH (AFTER ALLOXAN)	CAUSE OF DEATH
					First	Last		
	grams	mgm.	hours	hours	hours	hours	hours	
2	1925	185	24	2	2	7	more than 16	Hypoglycemia ?
3	1210	204	24	2½	2½		2½	Hypoglycemia
7	1920	200	24	17	5½	12	more than 17	Hypoglycemia
10	950	200	24	4	4	12	more than 16	Hypoglycemia ?
11	1190	200	24	3½	3½		3½	Hypoglycemia
12	1230	200	24	2	2		2	Hypoglycemia
15	1250	200	24	2½	2½	6	6	Hypoglycemia
16	1390	200	24	2	2	6	6	Hypoglycemia
17	1320	200	21		2	12	12½	Pulmonary edema
18	1185	200	21		2		3	Pulmonary edema
22	1415	180	none	3½	3½		3½	Hypoglycemia

by epinephrine on the pancreatic vascular system (5-8) in the rabbit. It should be pointed out that in the case of *rabbits no. 13* and *14* less than 20 minutes elapsed between the epinephrine and the alloxan injections as compared to 60 to 80 minutes with all other rabbits of our series. These 2 animals were among 3 (*no. 9, 13* and *14*) which, out of 11 that survived more than 24 hours, showed unmistakable diabetic hyperglycemia. Whether this is mere coincidence or whether an interval longer than 20 minutes between epinephrine and alloxan injections is necessary to prevent the diabetogenic action of the latter can only be decided by further research.

Fifty per cent of our rabbits died within 24 hours (table 3) and in most of these animals the cause of death was, in all probability, hypoglycemia. Four rabbits, *no. 3, 11, 12* and *22* died within three hours and fifteen minutes after the alloxan

injection and quite suddenly after the appearance of hypoglycemic convulsions, leaving no opportunity for effective treatment; two other rabbits, *no. 15* and *16* died six hours after the injection of alloxan in hypoglycemic convulsions, despite previous and repeated dextrose injections; two others died from pulmonary edema. The other three animals that died within 24 hours did so late in the night, 16 hours or longer after the alloxan injection. They had received several dextrose injections and the watch had been discontinued, since, in the light of previous experience from our own and other laboratories (2, 3, 4), it seemed that there should be no more danger of death from hypoglycemia.

Nearly all of our animals that died within 24 hours had been fasted for 21 to 24 hours and most of them were very young. We cannot rule out the possibility that these factors might have something to do with the high mortality from hypoglycemia, its early appearance and its late recurrence in our experiments. With this reservation we feel that the features of the hypoglycemia shown by these animals were a result of epinephrine-alloxan treatment as detailed above.

Low, and even very low, blood sugar figures were recorded in several animals 24 hours or more after the alloxan injection (table 1) as follows: *no. 8*, 62 at 24 hours; *no. 20*, 65 at 24 hours and 50 at 48 hours; *no. 6*, 37 at 24 hours; and *no. 14* (table 2), 38 as late as on the eighth day. There can be no doubt that this extremely late persistence or recurrence of hypoglycemia is a result of the epinephrine-alloxan treatment, since nothing similar has been reported as a result of simple alloxan treatment in rabbits or other species.

In rabbits *no. 1* and *5*, the epinephrine-alloxan treatment did not produce diabetes (table 2) but did produce alloxan hypoglycemia (table 1). This observation, which is the first of its kind to be reported, conflicts with Goldner and Gomori's statement (9) that alloxan hypoglycemia has never been observed in animals that survived and failed to become diabetic, and with the widely accepted view that alloxan hypoglycemia is due to insulin released from the islets of Langerhans in the course of their destruction under alloxan action. This view is also opposed by the fact that in our rabbits, *no. 11* and *12*, that died from alloxan hypoglycemia (table 3), the pancreas was found to be histologically normal. From the point of view of the mechanism of production of alloxan hypoglycemia, our results bear out Houssay's contention (10) that alloxan hypoglycemia is not due to insulin released from the damaged pancreas but is brought about by some extra pancreatic mechanism, probably as a result of alloxan injury to the liver.

Finally, out of 11 animals that survived 24 hours or longer, six (*no. 4, 6, 8, 13, 14, and 20*, table 1) died within 2 to 8 days. In all probability, the cause of death of these animals was not diabetes but liver and/or kidney damage. This means an exceptionally high mortality as a result of alloxan-induced damage other than diabetes. Therefore, while realizing that further study is required to reach any definite conclusion on this matter, we feel that a previous injection of epinephrine, under the particular conditions of the reported experiments, tends to increase the damage induced by alloxan in organs or tissues other than the pancreas and, as a result, tends to increase nondiabetic alloxan mortality.

SUMMARY

Intramuscular injection of epinephrine in doses of 200 micrograms per kgm. of body weight given 60 to 80 minutes before administration of diabetogenic doses of alloxan to rabbits either very markedly hindered or totally abolished the diabetogenic effect of alloxan in most cases. The mechanisms of this phenomenon and the bearing of some experimental factors upon it are briefly discussed.

The results of our experiments suggest that a previous injection of epinephrine tends to alter the effects of alloxan by favoring an earlier appearance, a greater severity and a later recurrence of hypoglycemia, and by increasing the incidence and the severity of, as well as the mortality from, alloxan-induced damage other than diabetes.

We wish to thank Dr. R. Barroso, to whom we are indebted for the histological study of the pancreas and other organs of our rabbits and Dr. S. Zubirán for his kind interest in our work.

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EXCRETION OF URINARY 17-KETOSTEROIDS BY THE MALE RABBIT

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Received for publication January 23, 1948

Zimmerman (1, 2) observed that ketosteroids react with *m*-dinitrobenzene in an alkaline medium to produce a red color. Subsequently, it was found that in diluted solutions the optical density of the color is proportional to the concentration of the 17-ketosteroids within rigid conditions of assay. The Zimmerman reaction has been applied with considerable success to assay the 17-ketosteroid content of human urine extracts. According to the available evidence the urinary 17-ketosteroids are metabolites derived from the adrenal cortex and testes (3, 4). There have been few reports in which the Zimmerman reaction has been applied to the urine extracts of experimental animals. A Zimmerman reaction applied to urine extracts of rats is included in a report by Lampton and Miller (5). Crude neutral urine fractions were assayed using an aqueous modification of the Zimmerman colorimetric reaction. Karnofsky, Nathanson, and Aub (6) have reported the 17-ketosteroid excretion in the mouse. From their report it appears that crude neutral fractions were used. Unfortunately, neither report present data to substantiate the methods used in their studies.

Since the nature and quantity of the urinary chromogens are unknown it was deemed important to test the applicability of the assay method for urine analysis of an experimental animal. The quantity of urinary 17-ketosteroids excreted by the male rabbit was studied, and data concerning the extent of correlation between volume and 17-ketosteroid excretion analyzed. Also, pathologies were induced in the adrenal glands and changes in the excretion of 17-ketosteroids determined.

MATERIALS AND METHODS

Eighteen mature male rabbits weighing 9.5–11.5 pounds were used. The colony was maintained under optimum conditions for at least six weeks prior to collection and assay of urine samples. They were fed complete rabbit pellets, and water was supplied in heavy stone crocks to avoid dilution of urine collections.

A quarter-inch mesh screen was placed beneath individual wire rabbit cages which sifted the fecal pellets but allowed the urine to pass through the mesh. Urine drained down a V-shaped tray tilted into a collection flask containing 4 cc. of concentrated HCl for preservation of the sample. Accurately timed 48-hour urine specimens were collected. Assays were made with 250 ml. aliquots of the total 48-hour collection. If the total urine collection was less than 250 ml. it was diluted to 250 ml. with distilled water. Urines were filtered and stored

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in the refrigerator until preliminary extraction which was completed within three days of the collection periods.

The crude neutral fraction. Urines were hydrolized by refluxing with 15 per cent by volume of concentrated HCl for seven minutes under 'cold finger' condensers. The hydrolized urine was cooled and immediately extracted four times with 35 ml. aliquots of ethyl ether. Combined ether extracts were washed once with water and twice with saturated Na_2CO_3 solution. Phenolic compounds present in the ether were removed by three washings with 2N·NaOH. Subsequently, the ether extract was washed three times with distilled water to remove alkalies. The ether solvent was then distilled off and the crude neutral urinary extracts dried and stored *in vacuo*.

Ketonic fractionation. The crude neutral fraction further treated with 40 mgm. of Girard's Reagent T according to the micro-method of Pincus (7) and a partially purified ketonic fraction obtained.

TABLE 1. 17-KETOSTEROIDS (MGM/250 ML.) DETERMINED FOR ALIQUOTS OF POOLED URINE AND COMPARED FOR PRECISION OF TECHNIQUE

ALIQUOT	POOL NO.							
	1	2	3	4	5	6	7	8
#1	5.8	6.5	5.8	2.5	5.2	1.3	5.5	6.2
#2	5.7	6.3	5.9	1.7	5.2	1.4	5.6	6.0
#3	6.1	6.7	6.2	2.0	5.1	0.9	5.2	5.9
#4	6.0	6.5	6.1	—	—	—	—	—
Average.....	5.9	6.5	6.0	2.1	5.2	1.2	5.4	6.0

Assay for 17-ketosteroids. An anhydrous modification of the Zimmerman colorimetric reaction developed by Talbot *et al.* (8) was used to assay the samples. For assay the urinary ketonic fraction was dissolved in 5.00 ml. of absolute ethanol. The optical density of the colorimetric reaction was measured in an Evelyn photoelectric colorimeter with a Rubicon filter having maximum transmission of 520 m. μ .

In order to evaluate the galvanometer readings of the urinary ketonic fractions the colorimeter was previously calibrated with six known concentrations of crystalline dehydroisoandrosterone² over a range of 50–300 γ . With each series of urine assays a standard was also assayed as a check on the technique. Each ketonic fraction, reagent blank, and standard was assayed in triplicate and the average of the three assays used as the representative value.

ANALYSIS OF THE METHOD

Overall precision for the technique was estimated from collections of rabbit urine pooled so that several 250 ml. aliquots were obtainable. The precision for comparable aliquots within eight such urine pools is presented in table 1.

² Crystalline dehydroisoandrosterone was kindly supplied to the investigator by Dr. E. Schwenk, Schering Corporation.

Accuracy of the method is reflected in the recovery efficiency of crystalline 17-ketosteroids added to urine. In such experiments 12 aliquots of pooled urine were assayed while 11 other aliquots of the same urine pool received measured amounts (5.00–6.00 mgm.) of crystalline androsterone. The average recovery was 88 per cent of the androsterone added to the urine.

The reagents were studied in several ways to examine their non-specific chromogen contribution. As a check on the constancy of the blank color with different batches of reagents it was routine to adjust the galvanometer to 100° and obtain a setting with the reagent blank. The blank setting during 10 months varied between 78°–82°. A scan was made of the colorimetric reagent blank absorption compared with an absolute ethanol blank in a Beckman spectrophotometer over a range of 400–640 m. μ . The resultant curve revealed maximum absorption at 450 m. μ . which rapidly decreased to a low level at higher wave lengths. Along with several urine samples a 250 ml. aliquot of distilled water was processed as if it were a routine urine collection. The water sample

TABLE 2. COMPARISON OF ASSAY VALUES FOR CRUDE NEUTRAL EXTRACTS AND KETONIC FRACTIONS (MGM/250 ML.) OBTAINED FROM ALIQUOTS OF POOLED URINE

URINE POOL	CRUDE FRACTION	KETONIC FRACTION	% CHROMOGEN AS KETONIC FRACTION
I	2.9	1.6	54.0
II	3.0	1.4	47.5
III	4.1	2.1	50.9
IV	4.0	2.1	54.0
V	3.0	1.6	53.7

showed no detectable assay value. Hence, it was assumed that the reagents used did not contribute chromogen which interfered with the assay method.

According to test experiments the technique of ketonic fractionation resulted in the largest loss of 17-ketosteroids. To determine the value of such purification in rabbit urine several aliquots of pooled urine were assayed in the crude neutral state while other aliquots of the same pool were purified to the ketonic stage. The assay values of the two fractions are compared in table 2. The average reduction in chromogen content is 52 per cent.

Optical density curves for the colorimetric reaction of various urine fractions and crystalline dehydroisoandrosterone were derived in a Beckman spectrophotometer at 20 m. μ . intervals from 400 to 640 m. μ . The crude neutral fraction of rabbit urine shows maximum density near 420 m. μ . and a second peak at 520 m. μ . The ketonic fraction has a single peak at 520 m. μ . and resembles the bell-shaped curve of crystalline dehydroisoandrosterone with the exception of relatively high density at 420 m. μ . The non-ketonic fraction has a peak at 420 m. μ and rapidly falls off in density at higher wave lengths. Such curves are roughly comparable to those obtained from the same fractions of human urine (9).

To determine the extent of non-specific chromogen the ratio of extinction co-

efficients $E_{520}:E_{420}$ was recorded for the fractions of rabbit urine. These ratios reveal that interfering chromogen is reduced by obtaining the partially purified ketones. A comparison of assay values with their respective extinction coef-

TABLE 3. AVERAGE 17-KETOSTEROID AND URINE EXCRETION PER 48 HOURS OF 18 NORMAL ADULT MALE RABBITS

ANIMAL	NO. OF URINES	MGM. 17-KS	S.E. $\pm \sigma_m$	ML. URINE
A	9	2.24	0.19	402
B	10	2.27	0.22	347
C	10	2.21	0.20	249
D	10	2.57	0.20	327
E	9	2.25	0.22	280
F	10	3.45	0.30	311
G	10	2.45	0.22	247
H	3	2.56	0.27	852
I	6	1.55	0.08	318
J	4	2.50	0.05	459
K	4	2.76	0.34	467
L	4	2.01	0.19	270
M	3	1.88	0.38	422
N	3	2.67	0.48	1080
O	3	1.91	0.23	283
R	4	1.78	0.05	284
S	4	1.28	0.15	343
T	4	1.93	0.12	287

TABLE 4. CONTINUOUS EXCRETION OF 17-KETOSTEROIDS (MGM/48 HRS.) ASSAYED FOR SEVEN NORMAL MALE RABBITS (FIGURES IN PARENTHESES ARE THE MEAN VALUES)

RABBIT A	RABBIT B	RABBIT C	RABBIT D	RABBIT E	RABBIT F	RABBIT G
2.03	1.50	2.50	2.69	1.72	1.96	2.30
2.76	3.32	3.17	3.91	1.35	2.58	2.31
1.72	1.42	1.20	2.41	1.69	3.14	1.81
2.48	2.12	1.85	2.33	2.08	3.24	3.42
2.28	2.45	2.52	2.01	2.88	4.79	2.81
2.14	2.42	2.53	1.82	1.95	5.14	1.65
—	2.72	1.35	3.01	—	4.35	2.27
3.38	3.49	2.90	3.28	3.44	3.44	3.96
2.11	1.71	2.37	2.35	2.76	2.90	2.15
1.31	1.56	1.69	1.85	2.51	2.95	1.80
(2.24)	(2.27)	(2.21)	(2.57)	(2.25)	(3.45)	(2.45)

ficient ratios suggests that as the concentration of 17-ketosteroids decreases the interference due to non-specific chromogen increases.

APPLICATION OF THE METHOD

In order to determine the excretion of 17-ketosteroids in normal adult male rabbits, 109 collections of 48-hour urine samples from 18 animals were assayed.

Table 3 summarizes the average 17-ketosteroid excretion for each animal of the colony. Individual rabbits vary in average excretion of 17-ketosteroids from 1.28 to 3.45 with the average excretion of the colony as 2.23 mgm/48 hours. Standard deviation of variates was ± 0.48 or 22 per cent of the mean value. Among 109 urine collections assayed the excretion of total 17-ketosteroids varied from 0.86 to 5.14 with a mean sample value of 2.31 mgm. per 48 hours and a standard deviation of ± 0.79 which is 34 per cent of the mean.

TABLE 5. AVERAGE 17-KETOSTEROID EXCRETION (MGM/48 HRS.) OF RABBITS IN NORMAL AND EXPERIMENTAL PATHOLOGIES INDUCED IN THE ADRENAL GLANDS

ANIMAL	NORMAL LEVEL	ME-CHOL. 305 DAYS	PERCENT DECREASE
D	2.57	1.95	25
E	2.25	1.59	30
F	3.45	1.62	50
G	2.45	2.28	7
		B-P TUMOR INVOLVEMENT	
W	2.31 ¹	0.33	86
X	2.31 ¹	0.18	92

¹ Excretion level of the colony.

TABLE 6. ANALYSIS OF COVARIANCE¹ OF URINE VOLUME ($X = \text{ML}/48 \text{ HRS.}$) AND 17-KETOSTEROIDS ($Y = \text{MGM}/48 \text{ HRS.}$) FOR SEVEN NORMAL RABBITS STUDIED FOR 9 OR 10 CONSECUTIVE COLLECTIONS OF 48 HOURS' DURATION

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES AND PRODUCTS			ERRORS OF ESTIMATE	
		Sx^2	Sxy	Sy^2	Sums of squares	Degrees freedom
Among rabbits.....	6	177237	6.68	4.5234		
Within rabbits.....	61	310217	2123.30	40.7034	26.1703	60
Total.....	67	487454	2129.98	45.2268	35.9196	66
Adjusted among rabbits.....					9.7493	6

¹ See, for example, *Statistical Methods*, G. W. Snedecor, Iowa State Press, 1940.

To determine the extent to which 17-ketosteroid excretion varies in individual normal animals the continuous urinary excretions of seven animals were studied for 20 days. Table 4 lists the excretions of these animals.

In an endeavor to alter the rate of adrenal function certain pathologies were induced which involved the adrenal cortical gland. Two black Dutch rabbits received inoculations of Brown-Pearce carcinoma suspension into the testes. Urine collections were begun seven days after inoculation. *Animal X* collapsed on the eleventh day so that two complete 48-hour urine collections were obtained. Three 48-hour collections were obtained from *animal W* before this animal succumbed. Although the collections were of normal urine volumes the 17-ketosteroid output was less than 0.5 mgm/48 hours in all samples (table 6).

Histologic sections of the adrenal glands exhibited numerous large metastases

of Brown-Pearce tumor cells. The rapidly proliferating carcinoma appeared to have replaced and crowded out the normal adrenal cells.

Another group of animals (*D*, *E*, *F*, *G*), whose normal 17-ketosteroid excretion level was established, were treated with 20-methyl-cholanthrene suspended in olive oil. Adrenals of each animal were exposed surgically, and 3-5 mgm. of methyl-cholanthrene deposited in each adrenal. Three hundred days later urine samples were collected continuously for eight days and assayed.

The level of 17-ketosteroid excretion was depressed in animals *D*, *E*, and *F*, while animal *G* excreted 17-ketosteroids at its pretreatment level (table 6). Histologic examination of adrenal sections revealed two unique cell types. Present in the adrenals of animals *D*, *E*, and *F* were many thin cords of very small intensely chromatic cells. These dark cell cords were very prominent immediately beneath the capsule of the cortex. They could not be identified as usual morphological constituents of the adrenal cortex. The second unusual cell type were clusters of highly vacuolated cells. These 'foam' cells were rare and found only in glands of animals *E* and *F*.

DISCUSSION

On the basis of data reported here concerning the preparation of rabbit urine extracts and their subsequent assay, the method has been demonstrated as being sufficiently quantitative, precise, and accurate enough to warrant application in animal studies.

The absorption spectra of various urine fractions indicate the ketonic fraction most closely resembles the curve of crystalline 17-ketosteroids, while fractionation experiments indicate that about half of the chromogen present in the crude neutral fraction is inert (table 2). In order to obtain extracts of sufficient chromogen purity it appears essential to obtain ketonic fractions. The excretion level of 17-ketosteroids in normal rabbits shows approximately the same purity of chromogen as the low level human urines reported by Talbot *et al.* (11).

It has been demonstrated that the principal 17-ketosteroids present in urine develop almost identical chromogen density in the relatively anhydrous assay modifications of the Zimmerman reaction (9, 10) but not with techniques using water as a reagent solvent. Since the constituent 17-ketosteroids of rabbit urine have not been characterized chemically, it appears advisable to use an anhydrous assay technique despite greater difficulty in preparation of alcoholic reagents.

To examine the relationship of urine volume to 17-ketosteroid excretion of rabbits an analysis of covariance was computed³ (table 6). The analysis was based upon a study of 48-hour samples collected continuously for 18 or 20 days on seven normal animals. There were 68 samples analysed for the correlation of urine volume with steroid content.

The data show statistically significant differences among the average urine

³ The analysis of covariance was performed by the University of Oregon Computation Service under the direction of Dr. W. J. Dixon, Department of Mathematics, University of Oregon.

volumes of the individual rabbits ($F = 5.7^4$ where $F_{.05} = 2.25$) while the differences among average 17-ketosteroid excretions for the seven individuals are not significant ($F = 1.13$ where $F_{.05} = 2.25$). Average correlation of the urine output to the 17-ketosteroid excretion on the basis of the seven individual animals is 0.60 and the correlation based on all the 68 urine collections is 0.45. The average values for urine volume and 17-ketosteroid excretion for the seven rabbits are not correlated. From such information it is obvious that excretion of 17-ketosteroids is relatively constant despite the variations in urine volumes.

To estimate the value of urine volume for predicting the 17-ketosteroid content a percentage comparison was made of the actual variance of y (mgm. 17-ketosteroid/48 hours) with the variance of y predicted on the basis of x (ml. urine/48 hours). The 'percent of information' or 'efficiency' of urine volume as an estimate of 17-ketosteroid content is $0.4362/0.6673 = 65$ per cent.⁵ The 17-ketosteroid excretion is considerably more constant when determined as a function of time duration rather than urine volume.

It is not known whether the depressed levels of animals receiving the Brown-Pearce carcinoma are due to a general debility of these animals near the height of metastases, or whether the resultant levels are directly attributable to the replacement and crowding out of active adrenal cells by the large metastases of tumor cells. The latter view is most adequately supported by histological studies of the adrenal sections.

The histological changes induced in the adrenals after 300 days exposure to methyl-cholanthrene do not appear to be typical adenoma of cortical cells but rather a proliferation of an anaplastic, densely chromatic type of cell. Since there were no other pathologies in these animals the depressed excretion of 17-ketosteroids is related to the changes in the adrenal glands. The experimentally induced pathologies of the adrenal gland suggests that interference with normal adrenal function lowers the excretion of 17-ketosteroids.

SUMMARY

An anhydrous Zimmerman reaction was applied to a partially purified neutral ketonic fraction of rabbit urine. The preparation of the fraction and its subsequent assay for 17-ketosteroids was studied to determine the applicability of the method used. The data of precision studies, recovery experiments, and studies of the chromogen purity indicate that the method is sufficiently accurate to warrant application utilizing the rabbit. In order to obtain fractions of sufficient chromogen purity it is essential to use ketonic fractions since the crude neutral fraction contains excessive chromogenic materials which yield false steroid titer.

The average level of 17-ketosteroid excretion in the normal male rabbit is 2.23 mgm. per 48 hours as indicated by assay of 109 urines collected from 18 animals.

$$^4 F = \frac{177237/6}{310217/61} = 5.7 \text{ (table 6).}$$

$$^5 26.1903/60 = 0.4362 \text{ and } 40.7034/61 = 0.6673 \text{ (table 6).}$$

A covariance study of the urine volumes excreted reveals that the excretion of 17-ketosteroids is relatively constant despite variations in urine volume.

Inoculation of Brown-Pearce tumor suspension into two animals reduced the excretion of 17-ketosteroids to less than 0.5 mgm. per 48 hours. The adrenal glands were invaded by large metastases of tumor cells. Methyl-cholanthrene deposited in the adrenal glands of four animals for 300 days induced histologic changes in the adrenal cortical gland. Three of these animals excreted a significantly lower level of 17-ketosteroids.

Depression of 17-ketosteroid excretion is correlated with changes in adrenal function as evidenced by the histology of the gland. Such evidence supports the adrenal source hypothesis of 17-ketosteroids.

The author wishes to thank Dr. Boris Krichesky for suggestions and criticisms made during the course of the study. I am grateful to Dr. Elly Jacobsen for the Brown-Pearce injected animals.

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INHIBITION OF EPINEPHRINE ACTION IN SEVERE HYPOXEMIA¹

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Received for publication January 21, 1948

Recently we have had occasion to observe the changes in blood pressure and heart rate in dogs in which the oxygen tension of the blood was reduced rapidly from normal values to a nearly absolute anoxemia. This acute form of severe hypoxemia was produced by 100 per cent nitrogen breathing. Under these conditions, the blood pressure may rise for a short period, as has been noted by several investigators (1-4). However, as the hypoxemia continues, the blood pressure falls progressively to low levels. Restoration of oxygen breathing causes a marked rise in blood pressure, considerably above the hypoxemia level.

We have found that epinephrine injected during the hypoxemic depressor phase fails to give the usual pressor response. Instead a reduced or absent response is seen as long as the hypoxemia continues. When oxygen is again introduced into the breathing mixture, the epinephrine then produces its pressor effect. This epinephrine action suggested a failure of responsiveness of the heart and blood vessels during extreme hypoxemia and indicated that the epinephrine could not be metabolized in the absence of oxygen, since a marked pressor effect appeared when the animal again breathed air. The lack of response to a potent agent such as epinephrine and its unexpected latency of action in severe hypoxemia led us to investigate the mechanisms involved. In this communication we report our results 1) on the blood pressure and heart rate changes following the induction of almost complete hypoxemia and after the subsequent reinstitution of air breathing and 2) on the response to the injection of epinephrine before, during and after the hypoxemic phase.

METHODS

Thirty-four dogs weighing from 6 to 17 kgm. were anesthetized with intravenous pentobarbital sodium (35 mgm/kgm.). Tracheotomy was performed and the vagi sectioned. Blood pressures were recorded from the femoral artery with a mercury manometer via ink writing on kymograph paper, or with the Hamilton manometer with optical recording of the pressure pulses. In 32 animals, the chest was opened by splitting of the sternum, or by incision in the fifth or sixth intercostal spaces with wide retraction of the ribs and removal of the mediastinal septum. Positive blast respiration was used.

It was found necessary to open the thorax to induce hypoxemia rapidly. Thus, when the positive pressure air respirator was disconnected from the tracheal cannula, the lungs collapsed and expelled most of their gaseous contents. Within

¹ Aided by the A. D. Nast Fund for Cardiovascular Research. The Department is supported in part by the Michael Reese Research Foundation.

a second or two, the tracheal tube was connected to positive blast from a nitrogen cylinder. On inflation with the nitrogen, the partial pressures of the gases in the lungs were probably now partitioned approximately as follows: a nitrogen tension of nearly 650 mm. Hg, a water vapor tension of about 50 mm. Hg (saturation at body temperature), an oxygen tension approaching zero, and the remainder carbon dioxide. Under these conditions, the circulating blood reaching the pulmonary capillaries quickly lost its oxygen via the lungs. Carbon dioxide was lost as in the normal animal. In this way we were able to set up a high oxygen gradient from the tissues to the lungs without accumulation of carbon dioxide and rapidly deplete the oxygen reserves of the animal. As indicated by the blood pressure response this rapid rate of deoxygenation did not obtain when the chest was not opened since the lungs did not collapse, and some oxygen remained in the alveoli. Also, by giving artificial respiration we were able to eliminate the

TABLE 1. TIME AND LEVEL OF THE MAXIMAL HYPOXEMIC BLOOD PRESSURE RISE

DURATION OF HYPOXEMIA	NUMBER OF TRIALS WITH HYPOXEMIC RISE	AVERAGE MAXIMAL RISE OBTAINED FOR EACH GROUP
<i>seconds</i>		<i>mm. Hg</i>
15	0	—
30	6	21
45	9	29
60	16	33
75	9	37
90	11	25
105	8	51
120	1	46
Total.....	60	

effects of respiratory failure which occurs commonly before the failure of the circulation.

At the end of periods of nitrogen breathing up to 180 seconds, the respirator tube was disconnected from the nitrogen tank and, after a second or two, reconnected to the air supply. This reversal of oxygen gradient caused a rapid return of the blood oxygen saturation to normal levels.

In some experiments epinephrine (0.5 mgm.) was injected into the femoral vein or into the left ventricle at various times, before and during the hypoxemic phase and also during the post-hypoxemic re-oxygenation phase.

RESULTS

1. *Effect of hypoxemia on the blood pressure.* Immediately after the institution of nitrogen breathing, in 60 out of 70 trials, a progressive rise in mean arterial pressure for periods varying from 30 to 120 seconds and averaging 70 seconds (hypoxemic pressor phase) was observed. The average rise observed was 33 mm. Hg, but in some instances it rose as much as 120 mm. Hg. The average data are given in table 1, and typical records in figures 1 and 4. No hypoxemic rise

was seen in 10 trials in 2 dogs which consistently failed to show this hypoxemic rise.

As the hypoxemia continued, the blood pressure leveled off, was maintained at its peak value for 15 to 45 seconds and then began to fall abruptly (hypoxemic depressor phase). This occurred usually after about 75 seconds of hypoxemia (range 30 to 120 seconds). Within 30 seconds, most of the blood pressures had already fallen to the pre-hypoxemic level. The pressure continued to fall to very low levels with circulatory failure and extreme dilatation of the heart occurring within a few minutes.

2. *Effect of re-oxygenation.* After 0 to 90 seconds in the hypoxemic depressor phase, air breathing was reinstituted in 65 trials. In 56 of these (90 per cent) the blood pressure was seen to rise sharply within the first 10 seconds. In most instances this pressor action produced values considerably above the control blood pressure level and even above the maximal pressure attained in the hypoxemic

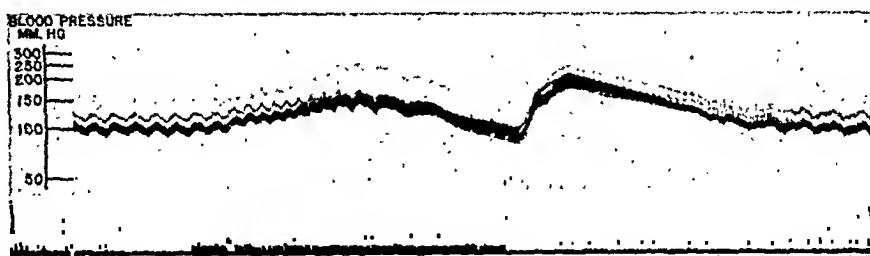


FIG. 1. EFFECT OF BREATHING 100 per cent nitrogen and reinstitution of air breathing in open-chested anesthetized dogs on the blood pressure recorded optically with the Hamilton manometer. Upper curve is blood pressure; lower curve, positive pressure respiration via automatic respirator (upstroke represents inspiration). Nitrogen breathing begun at break in respiration record at which amplitude of respiration line decreased. Air breathing resumed at break at which amplitude increased. Time, in seconds, recorded at bottom. Note the pressor effect early in hypoxemia, later giving way to a progressive fall in pressure. On resumption of air breathing, a marked pressor effect is seen.

pressor phase. The time of occurrence of the maximal pressure levels and their increase above the control level are shown in table 2.

3. *Relation between the duration of the hypoxemic depressor phase and the degree of the post-hypoxemic blood pressure rise.* A correspondence was found between the duration of the hypoxemic depressor phase and the degree of the post-hypoxemic rise. This is illustrated in figure 2 on data from three consecutive experiments. It can be seen from the curve that the post-hypoxemic pressor effect was minimal in animals subjected to short periods of hypoxemia (60 secs.) and in which no significant depressor phase was noted. In the animals in which a depressor phase was seen, i.e., those with a longer period of hypoxemia, the post-hypoxemic pressor effect was noticeably increased, and roughly in proportion to the duration of the depressor phase.

The duration of the post-hypoxemic pressor phase, measured as the time of return from the peak attained during the post-hypoxemic rise to the control pressure level, was in general a function of the magnitude of the rise above the previous level.

TABLE 2. TIME OF OCCURRENCE OF THE MAXIMAL PRESSURE LEVELS DURING THE POST-HYPOXEMIC PRESSURE PHASE

DURATION OF THE POST-HYPOXEMIC PERIOD	NUMBER OF TRIALS WITH MAXIMAL RISE ATTAINED IN EACH STATED TIME PERIOD	AVERAGE MAXIMAL RISE ABOVE CONTROL VALUES OBTAINED FOR EACH GROUP
<i>seconds</i>		<i>mm. Hg</i>
15	5	44
30	14	76
45	16	68
60	8	77
75	5	59
90	2	87
105	2	38
120	2	27

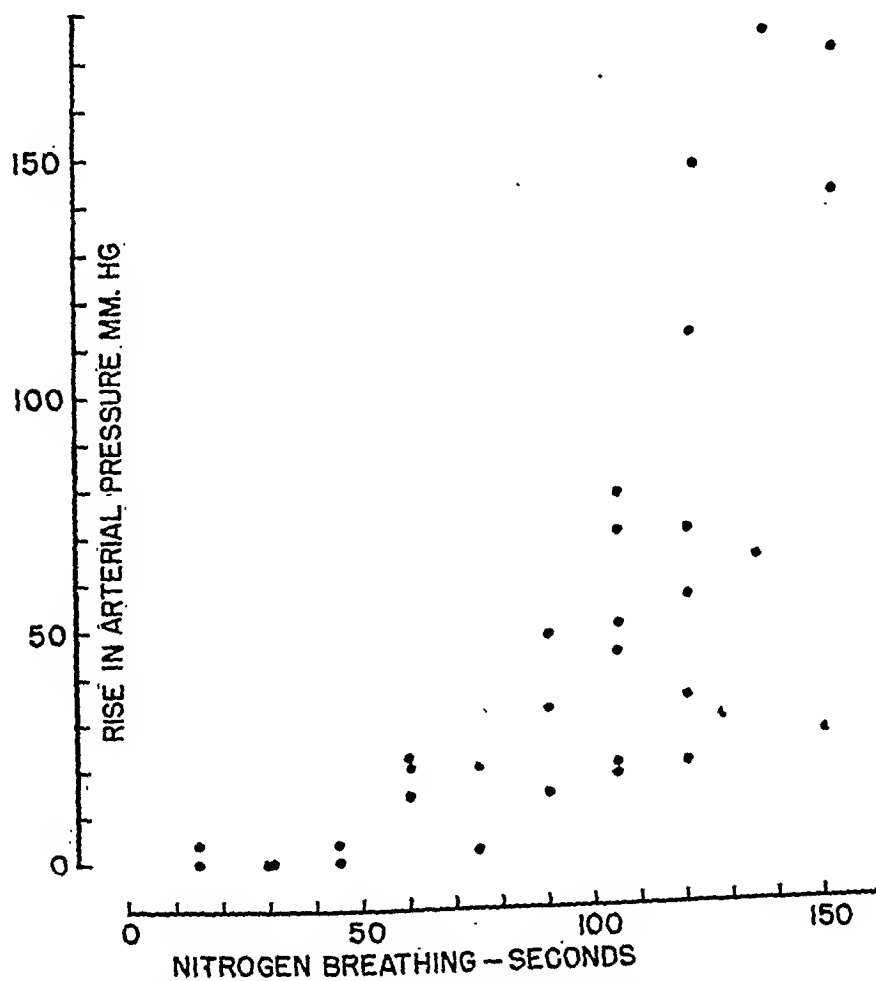


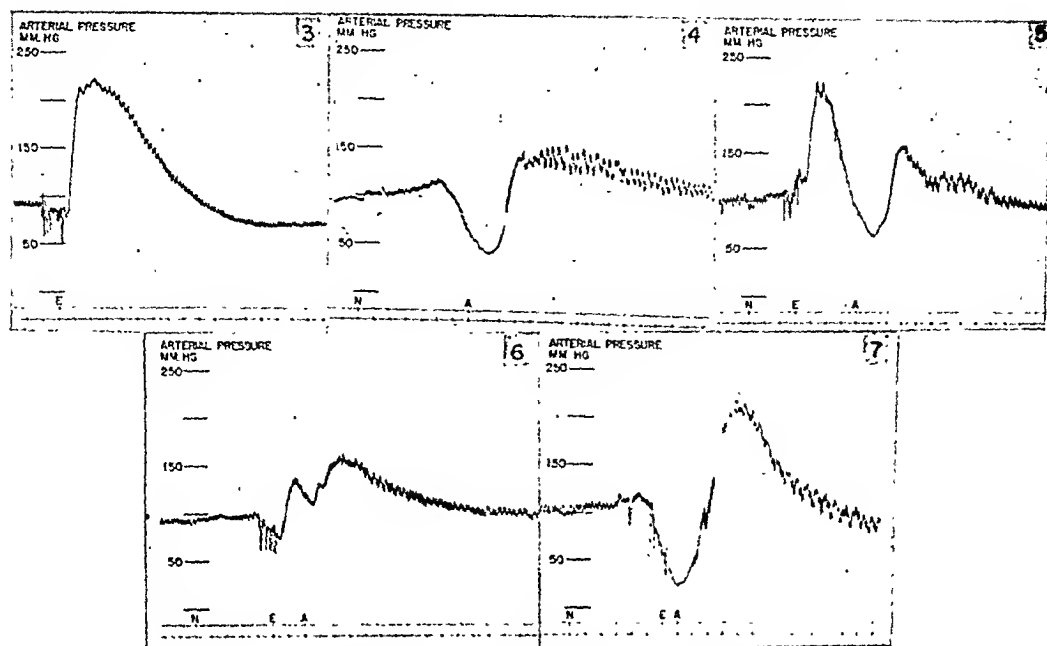
FIG. 2. SCATTERGRAM RELATING DURATION OF HYPOXEMIA to the post-hypoxic rise in arterial pressure. Abscissae give time in seconds of nitrogen breathing; ordinates, rise in blood pressure above control level in post-hypoxic period.

4. *Cardiac changes observed during the administration of nitrogen.* Shortly after the institution of nitrogen breathing, there was a tendency for the heart rate to accelerate, with a more forceful systolic contraction. As the blood pressure peak was reached during the hypoxemia period, the heart was observed to be beating vigorously and at a somewhat faster rate than originally. This continued into the phase of falling blood pressure. As the hypoxemia continued, the heart began to dilate and systolic contraction became weaker. In late hypoxemia, the heart was enormously dilated, contractions were feeble and A-V block supervened. When the hypoxemia was continued beyond this phase, the heart stopped in diastole.

5. *Cardiac changes observed upon the reinstitution of air breathing.* The cardiac changes observable upon the reinstitution of air breathing were dependent upon the stage to which the anoxia had been prolonged. When the heart had previously become dilated, with weak contractions at a slowed rate, a latent period (usually of 15-30 seconds) supervened following the resumption of air breathing. The heart rate then began to increase, contraction became more vigorous and the heart size decreased. In another 15-30 seconds the heart was beating vigorously and rapidly. During this period, the blood pressure increased rapidly toward its post-hypoxemic peak. The greater the duration of hypoxemia and the more severe the cardiac depression before resumption of air breathing, the longer was the latent period before recovery was seen. This effect can be seen in table 2. In several instances the hypoxemic depression of the heart was so profound that recovery did not take place with reinstitution of air breathing. Manual massage of the heart sometimes aided in recovery in such instances, although occasionally ventricular fibrillation developed as a result of this procedure.

6. *Effect of hypoxemia on the blood pressure response to epinephrine.* The usual effect of an injection of 0.5 mgm. of epinephrine into the femoral vein or left ventricle of the normal dog is a very rapid rise in pressure averaging 166 mm. Hg, with dissipation of the pressor effect in four or five minutes (fig. 3). When epinephrine was given at the onset of the hypoxemic period, the usual pressor response was seen, but the rise was not sustained as the animal went into progressive hypoxemia (fig. 5). When the epinephrine was given during the period of nitrogen breathing just prior to the time when the maximal pressure level was expected, the blood pressure would begin to increase but would fall again after a few seconds; i.e., the epinephrine pressor effect appeared to be aborted. During the early hypoxemic depressor phase, epinephrine injection resulted in an evanescent rise in pressure which quickly gave way to a fall in pressure even more marked than occurred normally. When the epinephrine was given in the late hypoxemic depressor phase, no change in the downward slope of the pressure curve was seen (fig. 7).

Although the pressor effect of the injected epinephrine was inhibited during the hypoxemic phase, it was again manifested after the reinstitution of air breathing. This was seen in the increased vigor of cardiac systole and in a greater than normal arterial pressure rise in the post-hypoxemic phase (compare figs. 5, 6 and 7



FIGS. 3 TO 7. SUCCESSIVE EXPERIMENTS ON THE SAME ANIMAL

FIG. 3. EFFECT OF LEFT INTRAVENTRICULAR INJECTION of 0.5 mgm. epinephrine on the blood pressure (*upper curve*) when animal was breathing air. Pressure recorded with a damped mercury manometer from the femoral artery. Calibration at left. Bottom line indicates time in 15-second intervals. Injection at E (this gives rise to irregularities of pressure curve preceding injection due to artefacts introduced by inserting needle into left ventricle). Following E there is an immediate rise in pressure reaching a maximum in about 20 seconds and falling off during the next few minutes.

FIG. 4. EFFECT OF NITROGEN BREATHING (N) and reinstitution of air breathing (A) on the blood pressure. Conventions as in figure 3. There is a slight rise in pressure following nitrogen breathing giving way after about a minute to a rapidly falling pressure. Air breathing is followed after a brief latent period by a marked pressor response above the control and hypoxemic pressure levels. The pressure then falls slowly to the control level.

FIG. 5. EFFECT OF EPINEPHRINE INJECTED, as in figure 3, during the pressor phase of the nitrogen-breathing period. Conventions as in figures 3 and 4. Epinephrine was injected during pressor phase of hypoxemia. It caused a less marked and less sustained pressure effect than in figure 3; within about 30 seconds the pressure rise was replaced by an abrupt fall in pressure, steeper than in the depressor phase of the control experiment in figure 4. Within approximately 20 seconds after resumption of air breathing the post-hypoxemic pressor effect appears; it is more marked than in the control experiment in figure 4.

FIG. 6. EFFECT OF EPINEPHRINE INJECTED, as in figure 3, during the early depressor phase of the nitrogen breathing period. Conventions as in figures 3 and 4. Epinephrine was injected during the depressor phase of hypoxemia. It caused a slight pressor effect, less marked and more transient than in figures 3 and 5 (compare with fig. 4). The post-hypoxemic pressor effect was more marked than in the control experiment in figure 4 and more protracted than when epinephrine was injected earlier during nitrogen breathing as in figure 5.

FIG. 7. EFFECT OF EPINEPHRINE INJECTION, as in figure 3, during the late depressor phase of the nitrogen breathing period. Conventions as in figures 3 and 4. Epinephrine was injected during the late depressor phase of hypoxemia. It caused no pressor effect during the hypoxemia period (contrast with figures 5 and 6). It may have had a masked depressor effect. A marked post-hypoxemic pressor effect occurred, greater than in the control experiment in figure 4 and also greater than in experiments where epinephrine was injected earlier during hypoxemia.

with fig. 4). In 43 trials in which epinephrine was injected into the femoral vein or left ventricle during the hypoxemic phase, a heightened post-hypoxemic rise averaging 136 mm. Hg and ranging from 39 to 192 mm. Hg above the reference level was seen. This compared with a post-hypoxemic rise of about 30 to 50 mm. Hg in those animals not receiving epinephrine. Six trials showed no increased post-hypoxemic rise after epinephrine. These six trials included four trials in which no recovery occurred and in which the circulatory failure was rapidly progressive, leading to death in a few minutes.

When epinephrine was injected early during the post-hypoxemic pressor period a very high pressure level was obtained without any significant latent period (see table 3), indicating a summation of the post-hypoxemic and epinephrine pressor effects.

TABLE 3. PRESSOR ACTION OF INJECTED EPINEPHRINE IN NORMAL, HYPOXEMIC AND POST-HYPOXEMIC STATES

CONDITION	NO. OF EXPERIMENTS	RESPONSE TO 0.5 MGM. EPINEPHRINE INJECTED INTO FEMORAL VEIN
Control.....	9	immediate rise, averaging 166 mm. Hg ¹
During early hypoxemic pressor phase.....	21	immediate rise, averaging 63 mm. Hg ¹
During late hypoxemic depressor phase.....	19	little or no response until return to air breathing after which pressor effect occurs in 15-75 seconds and averages 140 mm. Hg ¹
Post-hypoxemic phase.....	5	immediate rise, averaging 204 mm. Hg ¹

¹ Above control level.

In order to eliminate the influence of variations in venous transport to the heart, injections were made directly into the left ventricle at various times during nitrogen breathing in five animals. In these experiments, in contrast with femoral injection, some pressor activity could be demonstrated during the early hypoxemic depressor phase (fig. 6). In general, however, the arterial pressure responses to such intracardiac injections were similar to those observed with intravenous injection. Little or no pressor responses were obtained when epinephrine was injected into the left ventricle in the late hypoxemic depressor phase (fig. 7). Figures 3 to 7 illustrate the response to intraventricular injection of epinephrine in a single animal after varying periods of nitrogen breathing as compared to that after the injection of epinephrine during air breathing and as compared to the changes of hypoxemia alone.

DISCUSSION

We have presented a detailed study of the effects of acute severe hypoxemia on the blood pressure. The pressor effects seen during the hypoxemic and post-

hypoxemic period have been observed previously (1-4). Earlier studies have, for the most part, been carried out on animals in which hypoxemia was induced by the breathing of air with low oxygen tensions as in the decompression chamber with mixtures of gases. In none, however, has there been a quantitative analysis of the effects of the more fulminating form of hypoxemia which we have produced.

It is clear that rises in blood pressure occur fairly constantly during early hypoxemia. With our technique this phase lasts for only a minute or so after the institution of nitrogen breathing. Following this, the blood pressure begins to fall and this depressor phase, if permitted to continue, results in death within a few minutes. Reinstitution of air breathing after various durations of the hypoxemic depressor period results almost always in an immediate and marked blood pressure rise. This rise is usually greater than the pressor effect seen during the early phase of nitrogen breathing. Our results indicate that the degree of this post-hypoxemic pressor effect is related to the duration of the hypoxemic depressor phase.

Our results may be interpreted as indicating that the hypoxemia results in sympathetic stimulation and the liberation of a pressor material which manifest themselves at once in the rise in arterial pressure. The rise with early hypoxemia appears to be due mainly and perhaps wholly to sympathetic stimulation (4, 5). However, as the hypoxemia is continued, a point is reached at which the pressure begins to fall. This fall in pressure is apparently due to a failure of the circulatory system to respond to pressor states since subsequent re-oxygenation of the animal results in post-hypoxemic blood pressure rise. The magnitude of this rise in pressure is independent of changes in the heart rate although these may occur (fig. 1).

The fall from the hypoxemic peak is so rapid that the reference level is reached in about 30 seconds. While the rapid fall begins during a time when the heart is not grossly dilated and appears to be pumping vigorously, most authors attribute the fall solely to a diminished cardiac output (4, 6, 7). This point of view has been espoused on the basis of cardiometric and plethysmographic studies and analyses of pulse pressure contour curves, but it has not been concurred in by all (2). It is undoubtedly true that in the late stages of hypoxemia, heart failure is present as is indicated by the obvious marked dilatation and the diminished stroke volume. However, further attention must also be focused upon the failure of the peripheral blood vessels to maintain their tone as a possible participating mechanism. In favor of this viewpoint is a report that the isolated leg perfused with anoxic blood shows a vasodilatation which gives way to vasoconstriction when the leg is re-oxygenated (3). Further suggestive evidence is present in the work of Dale and Richards (8) who reported that histamine caused no vasodilatation in the presence of blood or of gum-Locke solution (low oxygen content) containing epinephrine, although it did cause vasodilatation when epinephrine and blood (higher oxygen content) were present together in the perfusing fluid. Because of the factor of cardiac failure which appears late in severe

hypoxemia the rôle of the peripheral vessels cannot be assayed with certainty from our data.

The post-hypoxemic pressor effect persists after section of the cervical spinal cord (4, 10, 11). Stavraký (4) has reported that a substantial post-hypoxemic rise appears in adrenalectomized animals. The relationship between the duration of the hypoxemic depressor phase and the degree of the post-hypoxemic rise which we found would indicate the accumulation of pressor material during the hypoxemic depressor phase which requires the presence of oxygen in the tissues for it to exert its pressor action. In the absence of oxygen this material does not lose its potential pressor effect, but accumulates so that its action is seen only after the re-oxygenation of the tissues.

During the early stages of the hypoxemic pressor phase, epinephrine caused a rise in pressure which was aborted with the development of a greater degree of hypoxemia. When the epinephrine was given intravenously during the hypoxemic depressor phase, no rise in pressure was observed until the reinstitution of air breathing. During increasing hypoxemia a progressive diminution of pressor response was seen. When oxygen was again made available to the tissues, the previously injected epinephrine manifested itself by a rise in pressure which was usually considerably greater than that seen in the control. In this way, the epinephrine acted to summate with the pressor material produced during the hypoxemic depressor phase.

Our results suggest that epinephrine requires the presence of oxygen in the blood stream and tissues in order to exert its pressor action. In the absence of oxygen, it fails to act, but apparently it is not destroyed at a significant rate. This dependence on oxygen for physiological action may be considered as supporting the contention of Blaschko and Schlossman (9), based on *in vitro* work with amine oxidase, that the degradation of epinephrine requires oxygen. However, it is not yet clear whether the fall in pressure during the hypoxemic depressor phase and the inhibition of the pressor action of epinephrine are due to, an inability of the substances to act, or upon the failure of the heart and circulation as a result of the severe hypoxemia produced.

Aside from their strictly physiological implications, our results are pertinent to the treatment of certain emergency conditions involving hypoxemia. It is common practice to inject epinephrine into the circulation or even directly into the heart in conditions of severe hypoxemia such as after drowning. Our results indicate that such injected epinephrine may fail to have the desired effect because of the low oxygen tension in the tissues. Further it appears that an epinephrine-like pressor material is elaborated during the period of hypoxemia and that this substance remains in the blood and tissues until adequate oxygen again reaches the tissues. The introduction of further pressor materials in such situations would appear to serve no useful purpose. Oxygenation of the tissues is demanded before such endogenous or exogenous pressor materials can exert their beneficial effects. Furthermore, the intravenous or intracardiac injection of epinephrine

in severe hypoxemia may be deleterious since both hypoxemia and epinephrine can predispose the heart to ventricular fibrillation.

SUMMARY

1. Acute severe hypoxemia produced by nitrogen breathing in the anesthetized dog caused a gradual rise in arterial pressure, reaching a maximal level in 60 to 90 seconds. Continuance of nitrogen breathing beyond this time is followed by a rapidly progressive fall in pressure to shock levels.

2. Re-oxygenation after a period of falling blood pressure results in a marked rise in pressure. This post-hypoxemic pressor effect is attributed to the accumulation of pressor materials which do not act in the absence of oxygen.

3. Epinephrine injected during the hypoxemic depressor phase acts similarly in that its pressor action is held in abeyance until the animal is re-oxygenated.

4. Some physiological and clinical aspects of these findings are discussed.

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BLOOD GLUTATHIONE IN THE BOVINE¹

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Received for publication February 20, 1948

Numerous reports suggest the significance of glutathione in the physiological and pathological processes of the human being and of various animals. Some normal blood levels of this compound in reduced form reported in the literature for various species are: human beings, 34.5 (1); horse, 32.6 (2); ass, 36.4 (2); mule, 36.1 (2); Bactrian camel, 24.2 (3); dromedary, 25.4 (3); hybrids of Bactrian camel and dromedary, 26.4 (3); and rabbits of the breeds Champagne, 47.6 (4), Flemish Giant, 40.0 (4), German Ermine, 45.6 (4), and scrubs, 43.7 mgm. per cent (4). According to one report (1) the normal content of oxidized and total glutathione in human blood is 13.5 and 47.3 mgm. per cent, respectively.

Although Harding and Cary (5) were able to demonstrate the presence of glutathione in the blood of the bovine, the blood levels in healthy cattle apparently have not been studied previously. In view of the suggested significance of glutathione in other species and the lack of data on the bovine, this study was initiated for the purpose of ascertaining the concentration of this substance in both sexes of healthy cattle of various ages and performing different physiological functions. The possible effect of diet upon the levels of glutathione in the blood of these animals was also investigated.

EXPERIMENTAL

Blood glutathione was measured approximately monthly for 15 months in 12 bulls constituting two groups in a feeding experiment and producing semen at varying rates, and in 49 cows and their 50 calves immediately following parturition. The cows composed four groups in an experiment designed to study the effects of various mineral supplements in the prepartal diet. Reduced, oxidized and total glutathione were determined in whole blood according to the method outlined by Woodward and Fry (6).

RESULTS AND DISCUSSION

Diet and blood glutathione. In a study of the relative merits of a simple and a complex concentrate feed mixture for breeding bulls, no differences were found in the reduced, oxidized and total glutathione content of the whole blood of bulls receiving equal quantities of digestible nutrients in these diets. Since the complex diet provided much greater quantities of total protein (from a variety of sources), crude fat, calcium, phosphorus, manganese, iron, cobalt, copper, vitamins A and D and other factors than did the simple mixture, a dietary effect

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upon the level of blood glutathione in the bull seems remote. Since dietary influences appeared to be lacking, the data for the two groups of bulls were combined and are shown in table 1.

In an experiment in which calcium and various trace elements were fed to cows for at least two months prepartum, these elements did not elicit perceptible effects upon the blood glutathione of cows or their newborn calves as determined in blood procured immediately following parturition.

Relation of age and colostrum ingestion to blood glutathione. The data presented in tables 1 and 2 demonstrate that the concentration of reduced and total glutathione

TABLE 1. RELATIONSHIP OF AGE OF BULLS TO BLOOD GLUTATHIONE LEVELS
Mean \pm std. error

AGE	NO. CASES	GLUTATHIONE (MG. %)		
		Reduced	Oxidized	Total
<i>mo.</i>				
18-21	24	28.10 \pm .73	5.74 \pm .42	33.81 \pm .78
21-24	35	30.71 \pm .90	8.40 \pm .34	39.11 \pm .85
24-27	47	34.93 \pm .72		
27-30	22	34.90 \pm 1.22		
30-33	33	41.51 \pm .93	6.34 \pm .45	47.11 \pm 1.34

TABLE 2. BLOOD GLUTATHIONE OF COWS AND THEIR NEWBORN CALVES
Mean \pm std. error

BOVINE	NO. CASES	GLUTATHIONE (MG. %)		
		Reduced	Oxidized	Total
All cows.....	49	38.29 \pm 1.26	6.79 \pm 0.82	45.08 \pm 1.28
All newborn calves.....	50	56.36 \pm 2.10	7.08 \pm 0.86	63.44 \pm 1.93
Newborn bull calves.....	26	55.53 \pm 3.64	6.09 \pm 1.20	61.62 \pm 3.13
Newborn heifer calves.....	24	57.19 \pm 2.14	8.07 \pm 1.23	65.26 \pm 2.28
Calves (no colostrum).....	43	57.82 \pm 2.12	7.21 \pm 0.96	65.03 \pm 1.78
Calves (colostrum).....	7	47.78 \pm 6.74	6.33 \pm 1.91	54.11 \pm 6.38

thione in the blood of the bovine varies with age, whereas the quantity of oxidized glutathione remains within fairly restricted limits. Since the blood of newborn calves (average age less than one hour) contains significantly greater quantities of both reduced and total glutathione than their dams (table 2) or the other animals studied (table 1), it would appear that these levels decline sometime between birth and 18 months of age. The data further indicate that an increase in the levels of these constituents accompanies aging of bulls from 18 to 33 months (table 1). The cows used in this study ranged from 29 to 155 months of age. When these animals were grouped according to age in 10-month intervals, no mathematically significant differences were found between groups. This observation would indicate that the levels of glutathione remain fairly

fixed at maturity or that the picture relative to the influence of age on the blood level of glutathione is obscured by the physiological changes occurring in the cow at the time of parturition.

The figures shown in table 2 for seven calves which had ingested colostrum before blood was obtained for analysis, at an average age of 4.5 hours, would indicate that both the reduced and total glutathione concentrations decrease rapidly following colostrum ingestion. Perhaps the greatest proportion of the decrease in glutathione concentration from birth to 18 months of age occurs during the very early life of the calf. Similar levels of reduced, oxidized and total glutathione were found in the blood of the newborn calf regardless of sex.

Effect of semen production upon the reduction of oxidized glutathione in blood by metallic zinc. It will be noted that no values for oxidized or total glutathione are given in table 1 for bulls 24 to 30 months of age. Although an attempt was made to measure these constituents, some factor(s) appeared to be present in the blood which precluded a successful determination. The difficulty seemed to be associated with a brownish discoloration of the filtrate, a lack of bubbling when zinc dust was added to the acid filtrate (during which process-oxidized glutathione is normally reduced, allowing its quantitative measurement) and, in a few samples, some slight oxidation of reduced glutathione. These symptoms suggested that the failure to reduce oxidized glutathione and, thereby, the failure to measure total glutathione were due to some fault of the zinc or acid used. The use of a number of different lots of zinc and sulfosalicylic acid and different inorganic acids, however, failed to remedy the condition. During this period (24-30 months of age) the bulls were yielding semen at fairly high rates (average per bull, 1.41 ml. per day). When these animals were rested, it was found that the total and oxidized glutathione could again be measured. Therefore, it appeared that some property of the blood during the periods of high semen yield inhibited the reduction of oxidized glutathione by zinc, although the possibility of hydrolysis of oxidized glutathione to cystine (7) cannot be eliminated. A similar condition was reported by Dohan and Woodward (8) and Woodward (9) for human blood. These workers (8) using electrolytic reduction showed that oxidized glutathione is not bound by the proteins of blood as suggested by Quensel and Wachholder (10) as the reason for the failure to recover oxidized glutathione in acid filtrates, following zinc reduction. Woodward (9) and Dohan and Woodward (8) proposed that the blood contributes something to the filtrate which leads to inhibition of zinc reduction. This difficulty appears to be obviated by electrolytic reduction (8).

SUMMARY

A study was made of the levels of reduced, oxidized and total glutathione in the blood of breeding bulls and in that of cows and their newborn calves immediately following parturition.

Although diet within the limits employed in this study did not influence the concentration of glutathione of either form in bulls' blood, the levels of reduced

and total glutathione gradually increased as age progressed from 18 to 33 months. Following parturition, similar levels of glutathione were found in the blood of cows ranging from 29 to 155 months of age.

Calcium fed two months prepartum as the only supplement and in conjunction with various trace elements in a basic dry-cow feed did not significantly affect the levels of glutathione of either state in the blood of the dams or their newborn calves as determined in blood taken soon after parturition. The blood of the calves, however, contained significantly greater quantities of reduced and total glutathione than that of their dams or of any of the animals studied. Calves having ingested colostrum before blood was obtained for analysis, at an average age of 4.5 hours, showed markedly lower concentrations of reduced and total glutathione than those which had not received this food. These data suggest a rapid decline in blood glutathione during the early life of the calf. Similar levels of glutathione were found in the blood of newborn calves regardless of sex.

Age, sex and diet did not appear to be related to the level of oxidized glutathione, as the concentration of this compound was similar for all animals studied. The failure to measure oxidized glutathione in the blood of bulls yielding large quantities of semen was attributed to changes occurring in the blood under these conditions which inhibit the reduction of oxidized glutathione by metallic zinc.

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EFFECT OF ADMINISTERING AGENIZED AMINO ACIDS AND WHEAT GLUTEN TO DOGS¹

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Received for publication January 8, 1948

During the past twenty-five years different theories have been suggested as to the cause of canine hysteria or so-called 'running fits.' A comprehensive survey of the problem, made by Hewetson in 1936 (1), revealed that no definite cause of the disease was known at that time. Arnold and Elvehjem (2) suggested that a deficiency might exist in the ration of dogs which developed fits. In 1944, however, Wagner and Elvehjem (3) demonstrated that running fits were due to a toxicity brought on when high levels of wheat gluten were fed to dogs. A report by Mellanby (4) showed that fits occurred when wheat flour was treated with a commercial bleaching agent, nitrogen trichloride (agene). Untreated flour fed as long as sixteen weeks caused no untoward reactions. Moran (5), Newell *et al.* (6) and Silver *et al.* (7), confirmed Mellanby's work and also showed that other food products such as casein, zein, wheat gluten, rye and gelatin can induce fits when they are treated with nitrogen trichloride.

Although the general symptoms of running fits have been recognized for a long time, no intensive investigations have been undertaken to determine if physiological changes take place when the dog develops the seizures. Arnold and Elvehjem (2) were unsuccessful in preventing the occurrence of fits when lysine dihydrochloride was administered intravenously. Supplements of thiamine hydrochloride given orally or intraperitoneally and oral supplements of choline chloride were found by Morgan and Groody (8) and Wagner and Elvehjem (3) to be ineffective in preventing fits. The latter group (3) and Mellanby (4) reported that a vitamin A deficiency did not cause fits and that supplements of the vitamin had no effect in the prevention or cure of the disease.

The experiments reported here were begun in an attempt to determine if any changes occur in the blood of dogs when they develop fits. Results obtained when agene-treated products were fed to dogs, previously 'sensitized' with agenized flour, are also included.

EXPERIMENTAL

Weanling mongrel dogs up to six months of age were used in these experiments. Each animal was dewormed, dusted with DDT, immunized against

¹ Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Wallace and Tiernan Company, Inc., Newark, New Jersey, and from The Wilson Company, Chicago, Illinois. We are indebted to Dr. H. K. Parker of Wallace and Tiernan Company and to Dr. Betty Sullivan of The Russell-Miller Milling Company for preparing the agenized amino acids.

distemper and then transferred to a basal control ration (6) before use in the subsequent experiments. The animals were kept in a heated, lighted room in individual heavy wire mesh cages, the floors of which were concrete and covered with wood shavings. Under these conditions it was possible to make close observations of each animal and to follow its daily food consumption.

The general procedure followed, during the experiments on blood physiology, was to maintain healthy dogs on the basal control ration for a week or more during which time 10 cc. blood samples were removed daily before feeding. Agenized wheat gluten was then substituted in the ration in place of casein. Blood samples were taken for several days after the onset of fits was first observed. As is shown in the tables it was often possible to obtain samples when a dog was in the convulsive state. At the end of each experiment, dogs which had received fit-producing rations were transferred to the basal control ration for a recovery period of at least a week. By this procedure it was possible to use each dog for several experiments.

All blood was drawn from the radial vein. The prothrombin time of the plasma was determined by the method of Campbell *et al.* (9) and was estimated by the number of seconds required for a clot to form in 12.5 per cent plasma containing known amounts of calcium and thromboplastin. The thromboplastin was prepared at regular intervals from normal rabbit brain. Blood samples for the determination of serum calcium obtained in a dry, unoxalated syringe were carried out according to the method of Clark and Collip (10). To prevent clotting of samples, oxalated or heparinized blood was used in all other analyses. Hemoglobin determinations were carried out by the method of Evelyn (11). Routine red and white cell counts were made and hematocrit values were obtained with Wintrobe tubes. Blood sugar values were determined by the method of Folin (12).

Supplements of calcium pantothenate, potassium, magnesium, selenium and treated and untreated amino acids were administered orally, intravenously or subcutaneously as described later in an effort to suppress or induce fits. Mixtures of the more insoluble amino acids were put in solution with 2N NaOH and adjusted to pH 7.3 with HCl. To 'sensitize' the dogs, rations were fed which contained agenized wheat gluten or flour. When fits developed the basal ration was substituted and the dogs were allowed to return to their normal state. Tests were then carried out to see if the sensitized animals developed fits when non-agenized protein products or amino acids were fed. At the same time, control dogs which had never received agenized materials were tested in a similar manner. All amino acid supplements were fed daily at a rate equivalent to the amount of that particular amino acid which would be present in 40 grams of wheat gluten. A dog normally consumes 200 grams of the gluten ration (which contains 40 grams of wheat gluten) per day.

In the water retention studies, dogs were kept in all-metal metabolism cages before the gluten ration was begun. Weights were recorded, measured amounts of water consumed, urine excretion, and food consumption tabulated daily.

RESULTS

In tables 1 and 2 data are presented which summarize the experiments made on the composition of the blood of the dogs. These animals were selected from

TABLE 1. EFFECT OF AGENIZED WHEAT GLUTEN ON THE BLOOD COMPOSITION OF DOGS

DOG	DATE	WT.	HEMO- GLOBIN	HEMATO- CRIT	RED BLOOD CELL	WHITE BLOOD CELL	Ca	GLUCOSE	PRO- THROM- BIN TIME	COMMENT
		kgm.	mgm. per cent	mgm. per cent	Mil/cmm.	No/cmm.	mgm. per cent	mgm. per cent	sec.	
128 Basal ration	3/31	5.30	11.07	32.0	2.17	8600	11.6	97	26	
	4/2	5.20	9.34	36.0	3.36	6120	10.6	108	26	
	4/4	5.30	9.34	34.5	3.25	6560	9.4	107	26	
	4/7	5.20	9.80	34.5	2.04	7200	12.3	105	22	Fits ob- served
	4/8	5.20	9.88	33.0	1.85	6240	11.9	105	23	
	4/9 ¹	5.20	9.88	34.0	2.43	5680	11.7		25	
132 Basal ration	2/11	4.05	9.26	26.4	3.42	6425	11.6	97	26	
	2/12	4.15	7.80	27.3	1.98	4560	10.5	103	28	
	2/13	4.05	9.26	27.8	3.85	4120	10.6	108	26	
	2/16	3.80	8.05	21.8	2.93	4700	11.9	104	24	Fits ob- served
	2/17 ¹	3.75	10.87	29.4	3.65	6000	12.3	105	26	
	2/19	3.85	9.06	23.7	2.88	6400	11.9	105	25	
137 Basal ration	3/20	5.10	9.88	27.0	2.12	8720	10.7	102	30	
	3/21	5.10	8.74	27.0	2.67	8000	10.5	104	27	
	3/22	5.15		28.5	2.02	6280	11.8	104	29	
	3/26	4.95	9.34	31.5	3.53	5680	11.7	90	37	Fits ob- served
	3/27	4.95	9.34	33.0	1.92	7360	10.1	81		
	3/29 ¹	4.90	7.48	26.5	2.38	5120	11.5	85	34	
136 Basal ration	2/16	2.05	8.68	22.7	3.21	10360				
	2/17	1.90	7.04	17.0	2.49	7920				
Gluten ration	2/19	2.10	7.90	17.5	2.38	7120				Fits ob- served
	2/21 ¹	1.90	8.88	21.6	3.59	9870				

¹ Bled during seizure.

a larger group of dogs tested and for the sake of brevity the results are presented as a representative sampling of the data obtained. No appreciable changes were found in the values for hemoglobin, hematocrit, red blood cell count, white blood cell count, calcium, glucose, prothrombin, phosphorus, sulfur or vitamin C. Vitamin C values tended to vary but no definite trend was indi-

cated. Even when blood was withdrawn during a seizure, there was no change beyond the normal limits.

TABLE 2. EFFECT OF AGENIZED WHEAT GLUTEN ON THE BLOOD COMPOSITION OF DOGS

DOG NO.	DATE	MAGNESIUM	POTASSIUM	PHOSPHORUS	SULFUR	VITAMIN C
Basal ration						
		<i>mgm. per cent</i>	<i>mgm. per cent</i>	<i>mgm. per cent</i>	<i>mgm. per cent</i>	<i>mgm. per cent</i>
21	8/14	1.8	15	38	1.4	0.75
242	8/14	1.3	14	34	—	1.10
243	8/14	1.9	14	36	1.5	1.10
Gluten ration						
21	8/23	2.9	40	41	1.6	0.40
242	8/23	3.3	26	32	1.3	1.40
243	8/23	2.6	18	39	1.4	0.75

TABLE 3. WATER RETENTION STUDIES OF DOGS MAINTAINED ON THE BASAL RATION (20 PER CENT CASEIN), AND ON A RATION CONTAINING 20 PER CENT WHEAT GLUTEN

DOG NO.	DATE	WEIGHT	WATER CONSUMED	WATER RETENTION
		<i>kgm.</i>	<i>cc.</i>	<i>cc.</i>
128	Basal ration	3/10	4.00	480
		11	3.85	600
		12	3.95	370
		13	4.20	620
	Gluten ration	18	4.65	950
		19	4.60	940
		20	4.75	900
		21	4.75	1000
137	Basal ration	3/10	4.45	670
		11	4.65	900
		12	4.70	490
		13	4.70	690
	Gluten ration	18	4.95	1000
		19	5.10	860
		20	5.10	710
		21	5.15	860

Results obtained for magnesium and potassium when dogs were fed agenized wheat gluten showed an increase of 22–166 per cent above the normal values. It will be observed that for each dog tested, there was a marked increase in these values when agenized gluten was fed.

In table 3 are summarized the results obtained with representative animals

in the water retention studies. No variation in the water balance was noted when agenized gluten was fed in comparison to the water balance observed when the animals were fed the basal ration. Although the absolute water consumption and water retention had increased somewhat by the end of gluten feeding, it must be remembered that young dogs were used and a gradual weight increase was evident in all animals.

Since large amounts of wheat are grown in areas where selenium is present in the soil, it was earlier considered that running fits might be due to a toxicity of this element. Anderson and Moxon (13) have reported that Se toxicity in dogs is not observed until it is injected at a level of 1.5–2.0 mgm. per kilo of body weight. Analyses of several samples of wheat gluten gave values for Se far below those causing toxic effects in dogs when a comparable amount of gluten was included in a ration. As calculated from the values of Se in the gluten ash, 0.2 mgm. of Se would be consumed by a dog in a five-day period while developing fits on a gluten ration. One dog was thus injected subcutaneously with 0.2 mgm. Se as Na_2SeO_3 . No toxic symptoms were observed during the next three days at which time one mgm. of Se per kilo was administered subcutaneously. The dog was normal the next two days. On the sixth day 2.0 mgm. of Se per kilo were given subcutaneously. Forty-five minutes later the dog was ataxic and its breath was heavy with a garlic odor characteristic of Se poisoning. The dog became progressively weaker and was found dead the next day. At no time were there any symptoms of running fits.

To determine if magnesium has any effect in preventing fits two dogs having seizures were given 120 mgm. Mg, as MgCl_2 , intravenously. Twelve hours later the animals were still showing fits and were then given one gram, each, of Mg subcutaneously. Convulsive seizures continued for three more days, followed by the death of one animal.

One dog which had several attacks of fits was given 100 mgm. of potassium, as KCl, intravenously. No decrease in seizures was observed and the procedure was repeated the following day. Partial blindness, characteristic of an advanced stage of fits, was still evident two days later. Ataxic hind legs also indicated that K has no therapeutic effect in running fits.

A dog with fits was given 15 mgm. of calcium pantothenate intravenously followed by a second injection of 20 mgm. the next day. Seizures and all typical symptoms of fits were observed the second and third day after treatment. No improvement with calcium pantothenate was indicated.

In view of the results obtained by Silver *et al.* (7), using agene-treated amino acids to induce running fits, it was decided to study the effect of both orally and intravenously administered amino acids. Two dogs, while on the basal ration, were given seven grams of non-agenized glutamic acid daily. The amino acid, dissolved in dilute HCl, was fed by stomach tube for three days. No supplements were administered the next two days. For the next three days, glutamic acid, treated mole per mole with agene, was fed by stomach tube at the rate of seven grams per day. Both animals remained normal throughout this experimental period.

The same procedure was used to test the effect of untreated and treated tyrosine when 1.25 grams of the amino acid were fed by stomach tube to two dogs. No toxic effects were observed in either animal.

One hundred grams of dl-methionine, treated with agene at 50 grams per kilo of methionine, were incorporated in two kilos of basal ration, at the expense of sucrose. One dog consumed 100 grams of this ration a day for twenty days without showing any symptoms of running fits. Methionine sulfoxide was then prepared according to the methods of Toennies and Kolb (14 and 15) and fed in the basal ration at 0.6 per cent to one dog for 10 days. No reaction was observed. On the eleventh day, 2.4 grams of methionine sulfoxide were injected intravenously into this animal. An electroencephalogram record was taken at frequent intervals for the next hour but no changes in the E.E.G., characteristic of fits, were recorded.

Two young 'non-sensitized' dogs were fed 100 grams of the basal ration per day, containing one per cent highly agenzized cystine, for a period of eight days. There was no evidence of running fits. A dog, previously 'sensitized' when fed the agenzized gluten, received by intravenous infusion a mixture of the following highly agenzized amino acids: three grams cystine, two grams methionine, two grams tryptophane. There were no fits during or after the test.

Mixtures of each of the following highly agenzized amino acids have been incorporated into the basal ration and fed for a period of ten days: 16 grams cystine and 16 grams valine, 16 grams cystine and 32 grams valine, 16 grams cystine and 7 grams serine, 16 grams cystine and 14 grams serine. Each mixture was fed to a 'non-sensitized' and to a 'sensitized' dog but in no instance were any fits indicated or observed.

Two dogs, one 'sensitized' and one 'non-sensitized' were each given daily intravenous injections of two grams of agenzized cysteine for a three-day period. No reaction was observed in either dog. Cystic acid, an oxidation product of cysteine, was given I.V. at the rate of 1.25 grams per day for two days to two dogs. Neither the 'non-sensitized' nor the 'sensitized' animals came down with fits.

One gram of agene-treated tryptophane was fed by stomach tube to a young dog. A second animal received two grams of the material by stomach tube. Both dogs remained normal.

A mixture of amino acids, containing the amino acids known to exist in wheat gluten, were heavily treated with agene and then incorporated in the basal ration at the expense of half the casein. This ration was fed to two dogs for 12 days without any evidence of fits. The amino acids consumed daily by each dog were equivalent to those present in 40 grams of wheat gluten. Repeated experiments have shown that if a dog consumes 80 grams of gluten, treated at 20 grams per cwt. with agene, the animal will consistently develop severe fits.

Five grams of glutathione were treated with 2 grams of agene and then incorporated into the basal ration at a level of 0.5 per cent. One dog fed this ration for eight days remained normal throughout the test.

DISCUSSION

Although there was some variation between dogs in the case of each blood component determined, the data clearly show that those changes observed were consistent for all animals. There was an increase in the blood magnesium of dogs which developed fits, but intravenous injection of the element into animals fed a fits-producing ration did not precipitate seizures. Orent, Kruse and McCollum (16) have reported that dogs with a magnesium deficiency exhibit hyperexcitability and convulsions. Potassium also increased in the blood when agenized gluten was fed but it would seem that although there is a notable increment of this element when a dog develops seizures, the high blood potassium is not important in running fits. Even intravenous injections of KCl to dogs exhibiting intermittent fits did not increase the frequency or severity of the seizures. Earlier, Melnick and Cowgill (17) showed that the blood non-protein nitrogen values of dogs were essentially normal before and after the administration of gliadin. They concluded that no significant uremia existed when dogs develop running fits. Results of the present study on the blood and on the water retention of dogs tend to support that conclusion.

Schaefer, McKibbin and Elvehjem (18) have described a sudden prostration, convulsions, and gastrointestinal symptoms in pantothenic acid deficient dogs. Calcium pantothenate in this case did not have a therapeutic effect when it was administered to dogs having intermittent seizures.

Silver *et al.* (7) have reported that running fits can be induced in dogs when the animals are first 'sensitized' by feeding agenized flour rations and then injected with agenized amino acids. It must be emphasized that the seizures only occurred, on injection of amino acids, after the dogs had first been on a fits-producing ration for periods up to two weeks. Dogs in such a condition might easily develop a convulsive seizure when any foreign material is injected into the blood stream. In 12 dogs fed a non-agenized flour ration and then injected with agene-treated amino acids, only 4 animals showed E.E.G. changes suggestive of a sub-clinical seizure condition. In no case were fits reported.

The amino acids, cystine and cysteine, reported by Silver *et al.* (7) to be active, have been fed by mixing in ration, by stomach tube and by I.V. injection without causing any indication of even a mild fit. Until clinical convulsions can be precipitated by the toxic product isolated from agenized flour, when fed orally, the identity of the 'running fits' factor must remain in doubt.

SUMMARY

No consistent or significant differences were observed between values for hemoglobin, hematocrit, red blood cell count, white blood cell count, prothrombin time, glucose, calcium, phosphorus, sulfur or vitamin C of the blood of dogs when fed agenized wheat gluten and when fed the basal ration. A definite increase in the blood magnesium and potassium was observed when the agenized wheat gluten was fed.

Selenium toxicity in dogs is not related to the condition of running fits. In-

jection of magnesium, potassium or calcium pantothenate had no effect in preventing or lessening the intensity of the seizures.

Various arogenized amino acids were fed as part of a ration, by stomach tube or intravenously, but none of the 28 animals tested developed fits as a result of this treatment.

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THE pH OF GASTRIC MUCOUS SECRETION^{1, 2}

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Received for publication February 27, 1948

Data at present available on the pH of gastric mucus are so scant that they contribute little to the sum of knowledge about the physicochemical characteristics of this exocrine secretion. Most of the reports (on material from dog, cat and human) state that it is alkaline to litmus, but a few investigators found an acid reaction to this indicator or even to Congo red. In patients with no free acid, Bucher (1) usually obtained pH's (colorimetric) in the range of 6.0 to 6.6, with an occasional value as high as 7.2. Mecholy, injected subcutaneously into two fasting patients, yielded secretion with a pH (electrometric) of 8.9 (10). When administered iontophoretically, however, this pharmacological agent yielded pH's no higher than 8.3, but only a few values were reported (9). In dogs, the pH of mucus secreted spontaneously from both pyloric and fundic pouches was reported in the range 7.0 to 7.5 by Ivy and Oyama (7); Lim and Dott (8) found the same range for pyloric pouch juice, whereas Takata (11) reported 7.4 to 8.0. Similar material from cats had a pH of 8.4 (colorimetric), as reported by Gamble and McIver (2).

In the present investigation we determined the pH of a large number of specimens of mucous secretion, obtained from pouches of the gastric corpus in dogs. More than 575 values were obtained from both 'spontaneous' and chemically stimulated secretion, thus permitting of a statistical evaluation of the variability manifest in the work of previous investigators. Analysis of the opacity, consistency, and columnar cell content of a majority of these same specimens has already been presented elsewhere (5, 6).

METHODS

Almost all of the specimens of mucus employed for this study were collected from 10 Heidenhain pouch dogs; 15 of them were obtained from three other dogs with gastric explants prepared from the central portion of the greater curvature. Hence, the data relate to secretion from the gastric corpus—not the pyloric region. No experiment was ever started unless the mucosa was free of obvious parietal secretion, as evidenced by a pH of 6.0 or greater. The technique for establishing this condition, as well as all other details of collection procedure, have been described in a previous paper (5). Experiments were performed with

¹ Preliminary reports of this work were presented before the Federation of American Societies for Experimental Biology in 1941 and 1943. [Hollander and Felberg (3); Hollander and Stein (4)].

² This investigation was supported in part by grants from The Altman Foundation of New York and Wyeth Inc.

the following stimuli: 1) gentle massage of the mucosa with a soft rubber catheter for 3 to 5 minutes; 2) distilled water saturated with ether (no greater than six per cent concentration at 30°C.); 3) five per cent aqueous emulsion of clove oil; 4) aqueous eugenol emulsions in concentrations as high as five per cent; 5) ethyl alcohol, 50 per cent; 6) isotonic NaCl solution, 0.17 N; 7) hypertonic NaCl solution, 0.5 N; and 8) distilled water. A set of data on 9) 'spontaneous' secretion was also included. These last specimens were obtained either during experiments without direct stimulation or during preliminary periods preceding the application of one of the aforementioned stimuli. It may be assumed that none of the stimulating fluids exerted any chemical influence on the pH values for the following reasons: a) the stimulating fluid was aspirated from the pouch almost completely before the first specimen of secretion was collected; b) this specimen was discarded whenever it contained any visible amount of the stimulus; c) the stimulating fluids were unbuffered in all cases.

Electrometric pH determinations were made with a glass electrode. In general, no more than three minutes were required to attain constant readings, and these were found to persist for at least 15 minutes when a small series of specimens was studied for this purpose. Multiple determinations on any one specimen agreed within a range of ± 0.02 pH, provided the specimens were shaken vigorously to effect homogenization before measurement. Without such shaking, duplicate determinations were liable to differ by as much as several tenths of a pH unit. After the reproducibility of this technique had been established, only single determinations were performed. Since we were concerned with the pH values of mucous secretion, which was presumably free of parietal secretion, all values above 4.00 were included in the tabulation for statistical analysis.

RESULTS

Combined data. A preliminary study of the frequency distribution for all 579 pH values, irrespective of stimulus, is presented in figure 1. The frequency graph is markedly skew, with a long tail on the acid side and a precipitous drop from 8.5 to the upper limit of the range, 9.2. The mean and standard deviations for the entire distribution are 7.65 and 1.08, respectively, but in view of the marked skewness of the graph, and the results of a breakdown of the data by stimuli, these two statistics possess no physiological significance. For purposes of analysis, the distribution was divided into two subranges at 6.8, where the flat tail of the graph terminates. It is noteworthy that more than 85 per cent of the values are in the upper subrange (table 1, line for combined data).

Data for individual stimuli. Next, the data for the total population were broken down according to stimulus, and the following statistics were calculated for each of these groups of data: the mean, its standard deviation (S.D.M.) as well as that of the distribution (S.D.), and frequencies in the two pH subranges. These statistics are also presented in table 1. The stimuli are arranged in order of increasing mean pH—except for one per cent and two per cent eugenol, for which the difference in concentration has greater statistical significance than

the difference in mean pH. Merely by inspection of the data it is apparent that the differences among these means are statistically significant in many instances,

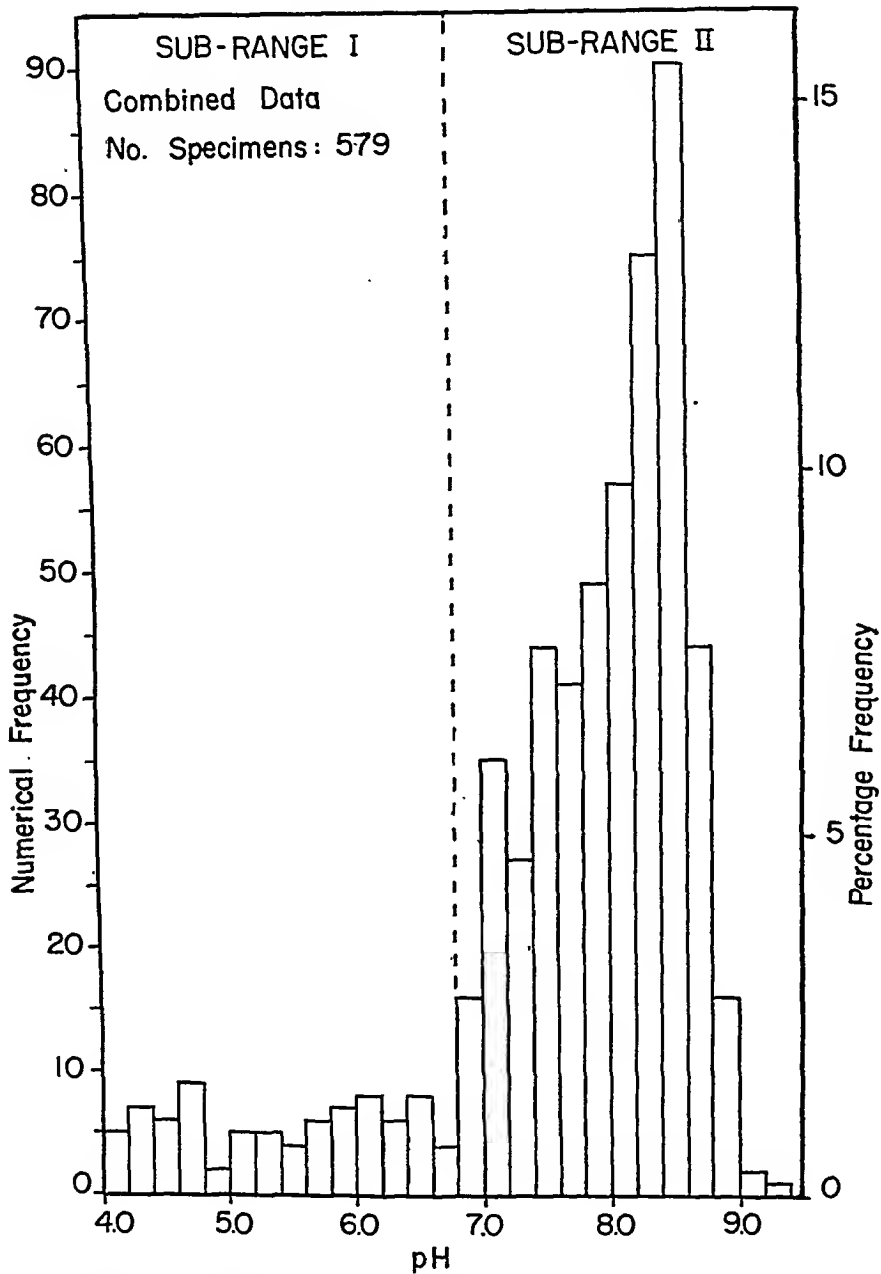


FIG. 1. FREQUENCY DISTRIBUTION for pH values of gastric mucous secretion from Heidenhain pouches (combined data—all stimuli).

but not in all. In nine out of 12 cases, the difference between two successive means is only 0.2 pH unit or less; in two others it is about 0.3 and in the third 0.4. Because of this small variation in mean pH from stimulus to stimulus,

TABLE 1. STATISTICAL DATA FOR pH VALUES OF GASTRIC MUCOUS SECRETION

STIMULUS	MEAN pH	S. D.	S. D. _M	pH RANGE	NUMBER OF SPECIMENS		
					Sub-range 1 (pH 4.00-6.79)	Sub-range 2 (pH 6.80-9.22)	Total
<i>Stimulus—group 1</i>							
Massage (mucosal)	6.48	1.24	0.28	4.20-8.58	12 (57.1%)	9 (42.9%)	21
NaCl (0.17 N)	6.66	1.25	0.38	4.08-8.43	4 (33.3%)	8 (66.6%)	12
H ₂ O (distilled)	6.83	0.96	0.30	5.19-8.36	5 (45.4%)	6 (54.6%)	11
None (spontaneous)	6.90	1.33	0.14	4.00-9.07	30 (31.6%)	65 (68.4%)	95
Alcohol (50%)	7.02	1.01	0.17	4.20-8.22	8 (21.1%)	30 (78.9%)	38
Eugenol (0.25%)	7.35	1.32	0.31	4.35-8.60	5 (26.3%)	14 (73.7%)	19
<i>Stimulus—group 2</i>							
NaCl (0.5 N)	7.40	0.44	0.13	6.38-7.95	1 (7.7%)	12 (92.3%)	13
Ether (saturated)	7.63	0.88	0.09	4.50-8.83	10 (9.3%)	97 (90.7%)	107
Eugenol (0.5%)	7.97	0.56	0.12	5.70-8.53	1 (4.3%)	22 (95.7%)	23
Eugenol (1%)	8.04	0.58	0.09	6.24-8.65	4 (8.7%)	42 (91.3%)	46
Eugenol (2%)	8.03	0.57	0.07	4.76-8.87	1 (1.7%)	58 (98.3%)	59
Clove oil (5%)	8.10	0.27	0.05	7.28-8.47	0 (0%)	27 (100%)	27
Eugenol (5%)	8.51	0.44	0.04	4.69-9.22	1 (0.9%)	107 (99.1%)	108
Combined data (all stimuli)	7.65	1.08	0.04	4.00-9.22	82 (14.2%)	497 (85.8%)	579

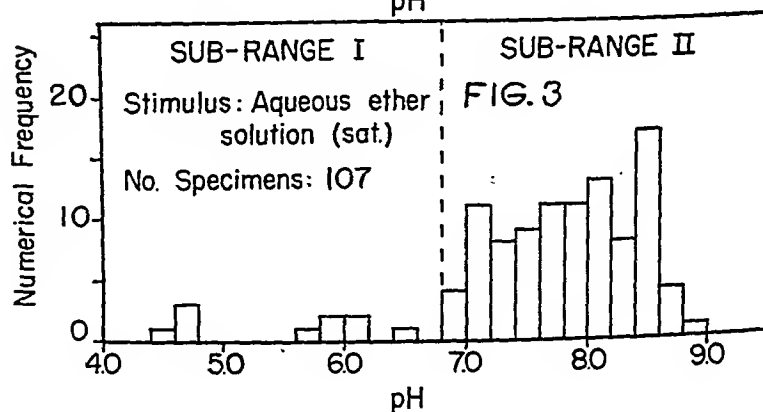
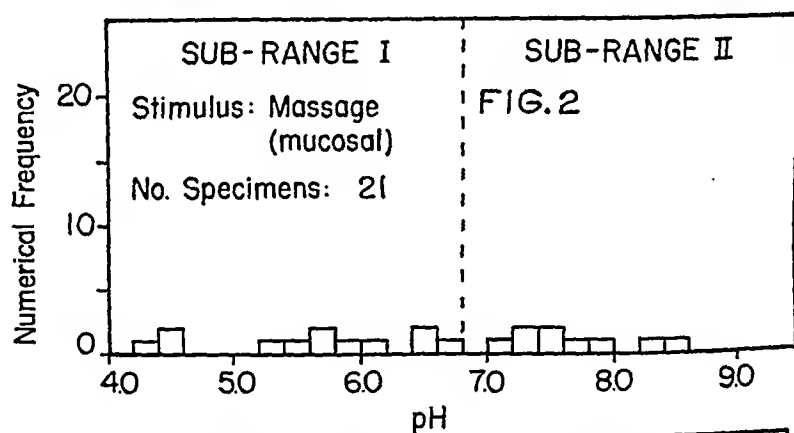


FIG. 2. FREQUENCY DISTRIBUTION for pH values of gastric mucous secretion from Heidenhain pouches (after stimulation of mucosa by massage).

FIG. 3. FREQUENCY DISTRIBUTION for pH values of gastric mucous secretion from Heidenhain pouches (after stimulation of mucosa with saturated aqueous ether solution).

these averages afford no basis for a classification of the stimuli. Such a classification, however, is suggested by the distribution of the data over the pH range. The values for mucosal massage, NaCl (0.17N), distilled water, 'spontaneous,' alcohol (50 per cent), and eugenol (0.25 per cent) extend more or less uniformly over the entire range. These are designated as stimulus-group 1. By way of illustration, the frequency graph for stimulation by mucosal massage is shown in figure 2. The data for the members of stimulus-group 2 show a well-defined concentration above pH 6.8, which is illustrated by the frequency graphs for ether, and eugenol (5 per cent) (figs. 3 and 4). For five of the stimuli in this group the number of specimens with pH's below 6.8 is no greater than one, and in the remainder the number does not exceed 10 per cent. In contrast with

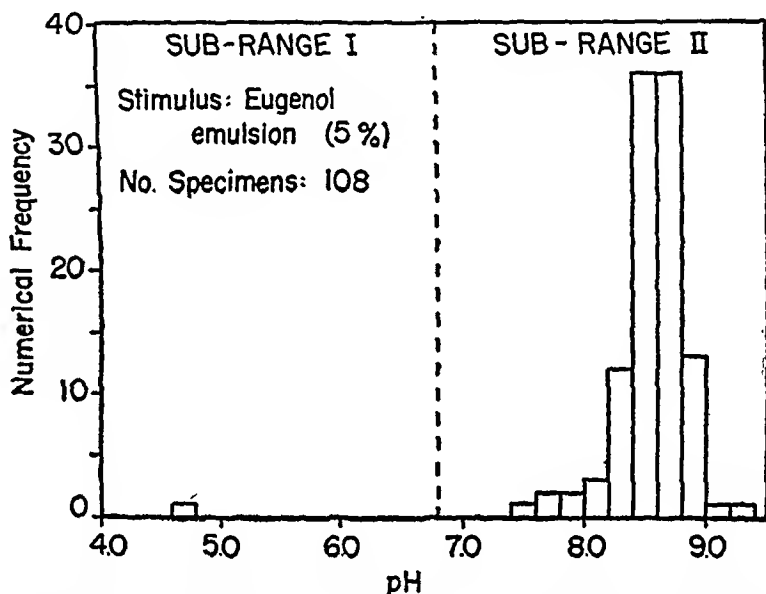


FIG. 4. FREQUENCY DISTRIBUTION for pH values of gastric mucous secretion from Heidenhain pouches (after stimulation of mucosa with 5 per cent eugenol emulsion).

this, the proportion of pH values below this boundary value of 6.8 among the stimuli of group 1 varies between 21 and 57 per cent.

DISCUSSION

The marked skewness of the frequency distribution for the combined data (fig. 1), particularly the long flat tail from pH 4.0 to 6.8, suggests the presence of at least two different types of physiological fluid in what is generally called 'mucous secretion.' The first, with a very low pH value, is probably parietal secretion; the second, with a very high pH value, is true mucus and possibly a mucoid secretion. The peaks at pH 7.1 and 7.4 might result from a third type of fluid, of intermediate pH, e.g., transudate or exudate, but they are hardly high enough relative to the peak at pH 8.5 to constitute evidence of a tri- or bimodal distribution. However, sufficient support for the presence of transudate and exudate is provided by our previous studies to warrant their inclusion as a third possible factor in the interpretation of the present data.

Hence, if we hypothesize that gastric mucous secretion is a mixture of these three type of fluid in varying proportions, then the lower subrange of pH can be considered as containing values characterized by the dominance of parietal secretion, and the upper subrange by the dominance of mucus and mucoid secretions. Inasmuch as the frequency curve for the combined data has only one well-defined mode (pH 8.5), and this occurs in the upper subrange containing 86 per cent of all the values, it may be inferred that mucus and mucoid secretion are by far the most common of all these components. The occurrence of transudate and exudate is more difficult to define; these probably exert a secondary effect in both subranges, but particularly in the lower portion of subrange 2, below 7.8. The role of desquamated columnar cells has been neglected in this hypothesis. It seems likely that in the nondisrupted condition these cells exert little if any hydrogen ion effect. When disintegrated, however, they contribute not only mucus but also cytoplasm, which may be assumed to resemble transudate in its influence on pH.

Turning now to the breakdown of the data by stimulus, it is apparent that every stimulus herein reported yields some 'alkaline' mucus, but the potencies of these agents differ considerably. Actual data for the stimuli in group 2 (from table 1) reveal that 91 to 100 per cent of the specimens induced by eugenol (0.5-5 per cent), clove oil (5 per cent), ether (saturated) and NaCl (0.5N), consist predominantly of true mucus, with a pH of 6.8 or greater. Small amounts of parietal secretion, transudate or exudate may also be present in these specimens. On the other hand the agents of group 1—massage, isotonic saline, distilled water, 'spontaneous' and eugenol (0.25 per cent)—are the poorest mucus stimuli inasmuch as only 43 to 79 per cent of their specimens fall in the upper subrange.

The present study relates to the pH of mucous secretion as it occurs inside a gastric pouch. Since by far the major part of the unoperated viscus contains corpus mucosa, it was deemed essential that the pouches be prepared entirely from this region, rather than from the cardiac or pyloric portions of the stomach. Ultimately, however, we are concerned with the whole stomach rather than any such limited portion of it, but evidence available from the literature suggests that pyloric mucus has essentially the same pH as that from the corpus.

Whether the observations herein reported cast any light on the pH of mucus as it is formed within the cell cannot be stated as yet, because no attempt was made to control or compensate for the possible loss of CO₂ from the specimen, either while it was being collected from the pouch or thereafter. An observation of Wright and Florey (12) is noteworthy in this connection. They reported that the pH of the viscous secretion of the cat's colon, resulting from faradic stimulation of the peripheral ends of the nervi erigentes, is at first 8.3 to 8.4 but increases to 9.1 to 9.2 on exposure to air. In the present work, it was noted that the pH of different portions of any one specimen become essentially identical if the specimen is well shaken. The uniformity so effected was ascribed to 'homogenization' of the specimen, but it may well be that the initial lack of uniformity was a result of irregular loss of CO₂ prior to shaking. Prevention of CO₂ loss before the specimen enters the collection tube is impossible with the usual procedures

for collecting the gastro-intestinal secretions. However, correction for such loss may be possible by equilibration of the specimen immediately before the pH is measured, using air containing CO_2 at its partial pressure in alveolar air. Such studies with gastric mucous secretion are now under way.

SUMMARY

Electrometric pHs were determined for 579 specimens of gastric mucous secretion collected from Heidenhain (corpus) pouch dogs. For this purpose, 12 topical stimuli were employed. The range of these pH values was 4.00 to 9.22, with a mean of 7.65 and a standard deviation of 1.08. Division of the data into two subranges of pH revealed that 82 (14 per cent) of the values fell in the lower interval (pH 4.00 to 6.79) and 497 (86 per cent) in the higher interval (6.80 to 9.22). It is inferred that the pH of this latter group is determined predominantly by true mucus, whereas the pH of the former group is influenced in considerable degree by the acid parietal secretion. Division of the data into 13 groups according to stimulus (including spontaneous secretion) revealed a gradual but extensive variation in the mean pHs for the individual groups. However, only aqueous emulsions of clover oil (5 per cent) and eugenol (0.5 to 5 per cent) yielded secretion with mean pHs greater than 8.0. Furthermore, 97 per cent of all the specimens obtained with these stimuli fall in the upper subrange, with pHs greater than 6.8. These observations support the conclusion previously reached by us that clove oil and eugenol are considerably more effective stimuli for the collection of gastric mucus than any others in the series.

The findings herein reported cast no light upon the pH of pure mucus as it is ejected by the surface epithelial cells, because of the loss of CO_2 from the specimen between the time of secretion and the time of pH measurement. Nevertheless, the data herein reported do indicate the pH which may be encountered in gastric pouches, and therefore presumably in the lumen of the intact resting stomach, under the influence of various mucus stimuli. This pH is usually above 7.4 and may even be greater than 9.0—probably the highest value encountered in the living mammalian organism.

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FURTHER STUDIES ON XANTHOPTERIN AND OTHER PTERIDINES IN BONE MARROW CULTURES

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Received for publication March 1, 1948

In 1936 Osgood and Muscovitz (1) cultured bone marrow *in vitro* using an elaborate setup, and Osgood and Brownlee (2) devised a simplified procedure for culturing the bone marrow cells. In 1947 Hays (3), using a modification of Osgood's simplified method, studied the reticulocyte production in bone marrow cultures. Plum (4) in 1947 studied the effects of liver preparations on bone marrow cultures using a modification of the original method of Osgood and Muscovitz (1).

Norris and Majnarich (5) have shown that xanthopterin will increase the cell proliferation in bone marrow cultures *in vitro*, with which a maximum is obtained with 5 γ of xanthopterin per ml. of bone marrow suspension for rat, rabbit, cat, sheep and beef bone marrow. In the experiments previously reported, a suspension of bone marrow cells in tyrodes without glucose was used. The only source of organic matter for the growth and metabolism of the cells would be that introduced with the bone marrow suspension. This would be small and might be a limiting factor in cell proliferation. As it was considered that the conditions used in the previous paper (5) were probably far from optimum, a study was made of some of the factors affecting the rate of cell proliferation in bone marrow cultures.

EXPERIMENTAL

The technique used was that outlined in a previous paper (5), in which 2 ml. of a bone marrow suspension in Tyrode's solution without glucose was placed in a sterilized 5 ml. vial. A glass bead was added to each vial. Supplements were added as indicated in the experiments, and the total volume in the vials was uniform for each experiment. The vial was sealed with a rubber cap and rocked in a water bath at 37°C. Samples for counting the cells were withdrawn at intervals by means of a sterile needle. Beef bone marrow was used in the experiments.

The amino acids were first studied as they might well be a limiting factor in production of new protein in cell proliferation. Figure 1 shows the results obtained for red blood cells (RBC), nucleated cells (NC), and reticulocytes. Combinations of tyrosine, tryptophane, and casein hydrolysate¹ were used. Addition of the various combinations produced a slight increase in the rate of cell proliferation, and all of about the same order of magnitude. Xanthopterin plus the

¹ The casein hydrolysate used was the 'vitamin-free' sterile 10 per cent solution of acid hydrolyzed casein manufactured by General Biochemicals, Inc., Laboratory Park, Chagrin Falls, Ohio.

amino acid combinations gave a still further increase in the rate of growth, and all were grouped in the order of magnitude of cell proliferation produced by xanthopterin without the amino acids, with the exception of the combination of

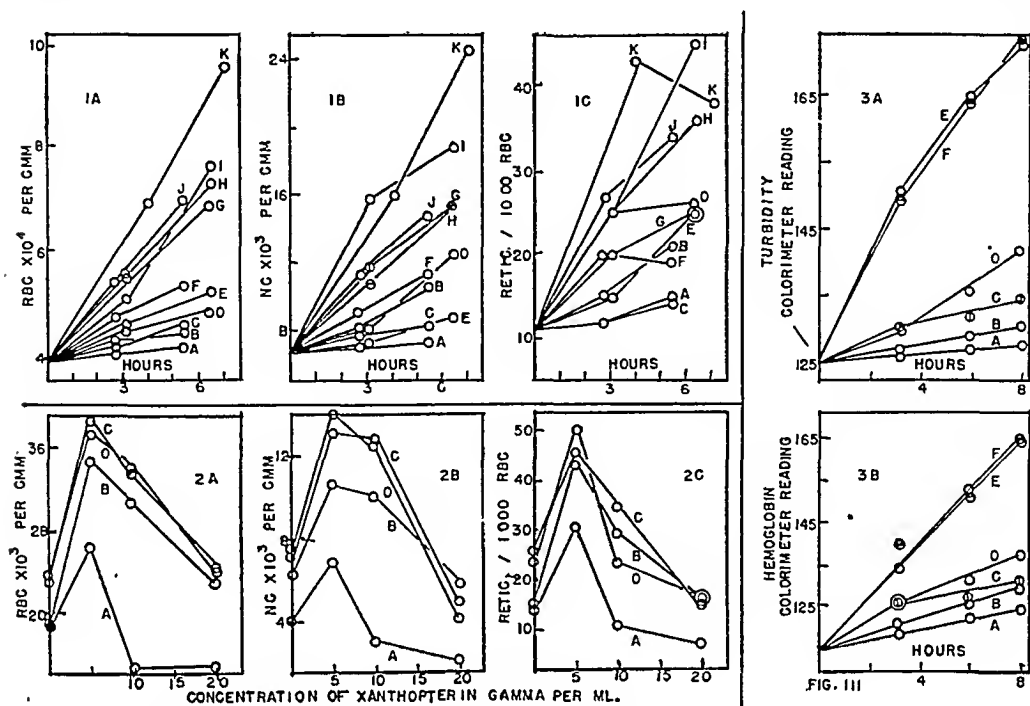


FIG. 1. EFFECT OF AMINO ACIDS AND CASEIN HYDROLYSATE upon the rate of cell proliferation in in vitro bone marrow cultures. The curves have the following supplements per ml. of suspension: A, no supplement; B, tyrosine, 1 mgm.; C, tryptophane, 0.5 mgm.; D, casein hydrolysate, 5 mgm.; E, casein hydrolysate, 5 mgm., and tryptophane, 0.5 mgm.; G, xanthopterin, 0.005 mgm., and tryptophane, 0.5 mgm.; H, xanthopterin, 0.005 mgm., and casein hydrolysate, 5 mgm.; I, xanthopterin, 0.005 mgm., tryptophane, 0.5 mgm., and tyrosine, 1 mgm.; J, xanthopterin, 0.005 mgm., and tyrosine, 1 mgm.; K, xanthopterin, 0.005 mgm., casein hydrolysate, 5 mgm., and tryptophane, 0.5 mgm.

FIG. 2. EFFECT OF VARYING CONCENTRATIONS of tryptophane upon the cell proliferation in bone marrow suspensions containing 10 mgm. of casein hydrolysate per ml. of suspension and varying concentrations of xanthopterin. The curves have the following concentrations of tryptophane: A, none; B, 0.1 mgm. per ml.; C, 0.5 mgm.; D, 1.0 mgm.

FIG. 3. HEMOGLOBIN FORMATION AND TURBIDITY ASSAY during cell proliferation of bone marrow suspensions. The curves contained the following supplements per ml. of suspension: A, none; B, xanthopterin, 0.005 mgm.; C, xanthopterin, 0.005 mgm., and casein hydrolysate, 5 mgm.; D, xanthopterin, 0.005 mgm., and tryptophane, 0.5 mgm.; E, xanthopterin, 0.005 mgm., casein hydrolysate, 5 mgm., and tryptophane, 0.5 mgm.; F, xanthopterin, 0.005 mgm., casein hydrolysate, 5 mgm., tryptophane, 0.5 mgm., and tyrosine, 1 mgm.

casein hydrolysate and tryptophane either with or without tyrosine. Tryptophane would be lacking in the casein hydrolysate produced by acid hydrolysis, and the addition of tryptophane to the hydrolysate would furnish all of the amino acids required for the production of protein.

The buffer in the Tyrode's solution was not sufficient to control the pH on the

addition of the casein hydrolysate. The hydrolysate had to be neutralized before adding it to the culture and was adjusted to a pH of 7.4 for all tests. However, a final pH varying from 7.0 to 8.0 showed no appreciable variation. The effect of varying the concentration of both casein and tryptophane was studied.

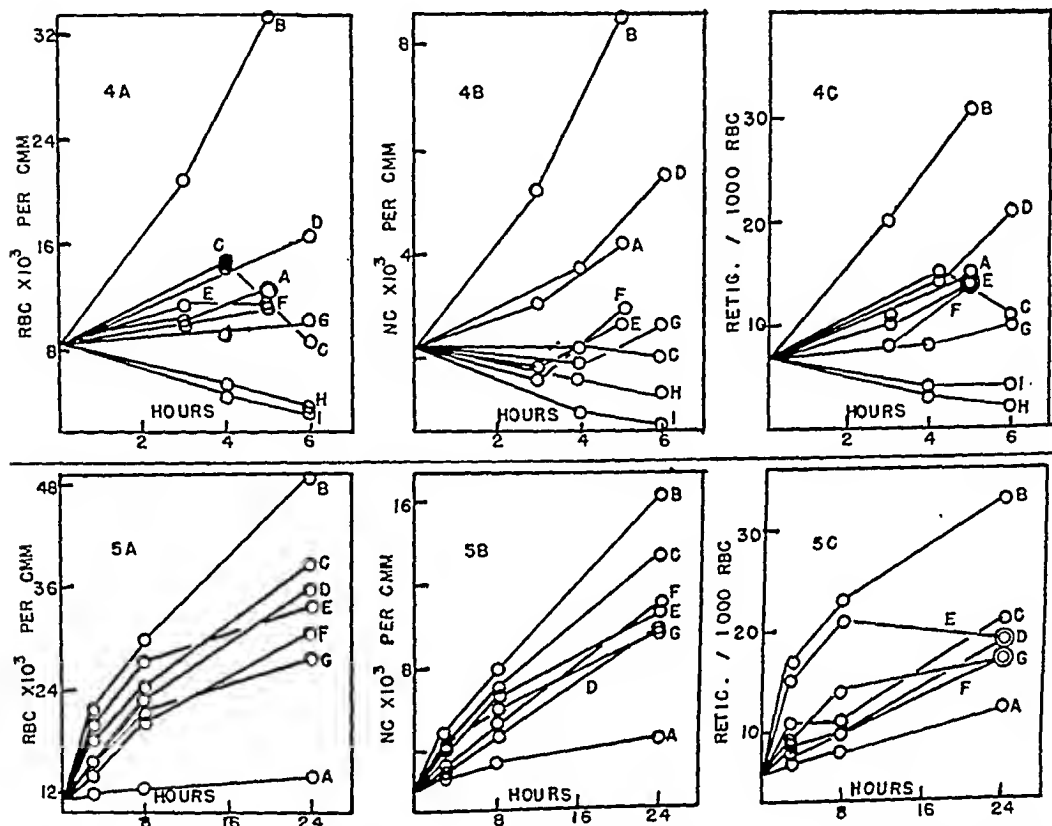


FIG. 4. EFFECT OF VARIOUS PTERIDINE DERIVATIVES ON bone marrow cultures in vitro. The curves have the following supplements, each at a concentration of 0.005 mgm. per ml.: A, none; B, xanthopterin; C, isoxanthopterin-6-carboxylic acid; D, 6-methyl-isoxanthopterin; E, 2-amino-4-hydroxypteridine·HCl·H₂O; F, dihydroxanthopterin; G, 7-methyl-xanthopterin; H, 2-amino-4-hydroxy-7-methyl pteridine; I, xanthopterin-7-carboxylic acid.

FIG. 5. EFFECT OF VARIOUS PTERIDINE DERIVATIVES ON bone marrow cultures in vitro. The curves have the following supplements, each at a concentration of 0.005 mgm. per ml.: A, none; B, xanthopterin; C, 2-amino-4-hydroxy-6-methyl pteridine; D, pterioic acid; E, 2-amino-4-hydroxy-6-pteridyl methyl pyridinium iodide; F, leucopterin; G, 2-amino-4-hydroxy-pteridine-6-carboxylic acid.

A concentration of 0.1 ml. of neutralized casein hydrolysate, equivalent to 10 mgm. of casein per ml. of suspension, was selected for all subsequent experiments. Figure 2 shows the response in cell proliferation with varying amounts of tryptophane using the concentration of casein indicated above. From the results a concentration of 0.5 mgm. of tryptophane per ml. of bone marrow suspension was adopted for use. While some variation has been observed between bone marrow preparations, in general the addition of casein hydrolysate and trypto-

phane not only increases the rate of cell proliferation due to xanthopterin but also broadens the optimum region of concentration of xanthopterin for cell division.

Erythrocyte formation would necessitate hemoglobin formation, which was studied along with the cell counts. A series of test tubes were prepared containing 6 ml. of bone marrow suspension and supplements as indicated in figure 3. A glass bead was added to each test tube, which was sealed with a rubber cap and rocked slowly in a water bath at 37°C. After incubating for intervals of 3, 6 and 8 hours, suspensions were transferred to colorimeter tubes and turbidity read on the Klett-Summerson photoelectric colorimeter. In a uniform suspension of cells the nephelometric reading is indicative of the number of cells. The results are very similar to those indicated by the counts given in figure 1. The

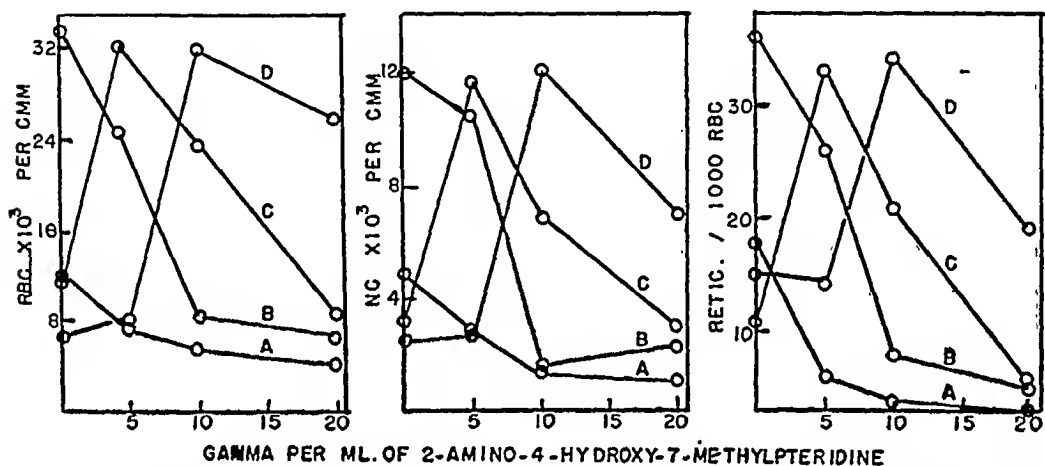


FIG. 6. INTERRELATIONSHIP OF XANTHOPTERIN and 2-amino-4-hydroxy-7-methyl pteridine upon the cell proliferation in bone marrow cultures. The curves represent the following concentrations of xanthopterin per ml. of suspension: A, none; B, 0.005 mgm.; C, 0.010 mgm.; D, 0.015 mgm.

cells were then completely laked and hemoglobin determinations made by the alkali method. The results are plotted as colorimeter readings. The increase in hemoglobin is shown to parallel the cell proliferation given in the experiments above and to be greatest when xanthopterin, casein hydrolysate, and tryptophane were added, either with or without added tyrosine.

The above experiments have shown that xanthopterin increases the rate of cell proliferation in bone marrow cultures in vitro. Several other pteridine derivatives have been tested on in vitro bone marrow cultures and none found with an activity as great as that of xanthopterin. Figures 4 and 5 show representative results. Casein hydrolysate and tryptophane were included in the culture media. 2-amino-4-hydroxypteridine \cdot HCl \cdot H₂O has little or no effect on RBC formation. Those compounds substituted on the six carbon atom stimulate both red and nucleated cell proliferation, such as 2-amino-4-hydroxy-6-methyl pteridine, 2-amino-4-hydroxy-6-pteridyl methyl pyridinium iodide, 2-amino-4-hydroxy-6-carboxylic acid, leucopterin, which is probably converted

to xanthopterin, and pterioic acid; however, none of them is as active as xanthopterin. The activity of pterioic acid is of interest compared to pteroyl-glutamic acid which is entirely inactive. The disubstituted compounds on the 6 and 7 carbon atoms have slight effect on erythrocyte proliferation in vitro and a somewhat greater inhibitory effect upon the nucleated cells. Compounds used were 6 methylisoxanthopterin, isoxanthopterin-6-carboxylic acid, 7 methyl-xanthopterin, and dihydro-xanthopterin. The two compounds that were distinctly inhibitory and acted as antixanthopterins were 2-amino-4-hydroxy-7-methyl pteridine and xanthopterin-7-carboxylic acid.

The antixanthopterin effect of 2-amino-4-hydroxy-7-methyl pteridine is shown in figure 6. The effect upon cell proliferation appears to be a remarkable balance between xanthopterin and 2-amino-4-hydroxy-7-methyl pteridine. Without added xanthopterin, 2-amino-4-hydroxy-7-methyl pteridine causes a decrease in the number of cells. This inhibitory effect is reversed by adding xanthopterin. We have found repeatedly that if equal concentrations of both are present the rate is not changed materially from what it is with no supplement, but when either is added in excess the effect of the substance in greater concentration becomes evident. The toxic or inhibitory effect of concentrations of xanthopterin greater than 5 γ per ml. is counteracted by added 2-amino-4-hydroxy-7-methyl pteridine. With the addition of increasing amounts of 2-amino-4-hydroxy-7-methyl pteridine the optimum concentration for xanthopterin activity is shifted to such an extent that there would be 5 γ per ml. of xanthopterin in excess in the mixture. That is, for zero 2-amino-4-hydroxy-7-methyl pteridine the optimum is at 5 γ per ml. of xanthopterin; with 5 γ per ml. of 2-amino-4-hydroxy-7-methyl pteridine it is at 10 γ per ml. of xanthopterin and at 10 γ per ml. of 2-amino-4-hydroxy-7-methyl pteridine it is at 15 γ per ml. of xanthopterin. Fifteen γ per ml. of xanthopterin is greater than the optimum concentration and inhibitory but in the presence of 10 γ per ml. of 2-amino-4-hydroxy-7-methyl pteridine gives an activity equal to that of 5 γ per ml. As the molecular weights of the two compounds are very close it appears to be a very surprising stoichiometric relationship between them in control of the cell proliferation.

SUMMARY

1. Amino acids and especially a combination of casein hydrolysate and tryptophane further increase the acceleration of cell proliferation in bone marrow cultures in vitro, produced by xanthopterin.
2. Of several pteridine compounds tested, none showed as great an acceleration of bone marrow proliferation as xanthopterin.
3. Leucopterin, pterioic acid, 2-amino-4-hydroxy-6-methyl pteridine, 2-amino-4-hydroxy-6-pteridyl methyl pyridinium iodide and 2-amino-4-hydroxy-pteridyl-6-carboxylic acid have some activity in stimulating cell proliferation in bone marrow suspensions.
4. Xanthopterin-7-carboxylic acid and 2-amino-4-hydroxy-7-methyl pteridine are strongly inhibitory to cell proliferation in bone marrow cultures.

5. The inhibitory action of 2-amino-4-hydroxy-7-methyl pteridine is counteracted by xanthopterin.

6. The inhibitory action of high concentrations of xanthopterin is counteracted by 2-amino-4-hydroxy-7-methyl pteridine.

The authors wish to gratefully acknowledge and thank the Wellcome Research Laboratories for the generous supply of dihydroxanthopterin, 2-amino-4-hydroxypteridine·HCl·H₂O, 6-methyl-isoxanthopterin, 7-methyl xanthopterin, isoxanthopterin-6-carboxylic acid and xanthopterin-7-carboxylic acid used in our experiments.

The authors wish to gratefully acknowledge and thank the Lederle Laboratories, Division of the American Cyanamid Company, for the generous supply of 2-amino-4-hydroxy-6-methyl pteridine, 2-amino-4-hydroxy-7-methyl pteridine, 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxy-6-petridyl methyl pyridinium iodide used in our experiments.

The authors wish to gratefully acknowledge and thank Merck and Co., Inc. for the generous supply of pteric acid used in our experiments.

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REDUCTION OF CENTRAL HYPER-IRRITABILITY FOLLOWING BLOCK ANESTHESIA OF PERIPHERAL NERVE

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Received for publication February 28, 1948

The purpose of this study was to determine whether hyper-irritability of the dental innervation, produced by previous painful stimulation of the teeth, could be permanently reduced to a normal level of irritability by a single application of procaine block.

This question arose from two previous studies (1, 2). In the first (1) it was observed that the most prevalent cause of dental pain engendered by alteration of barometric pressure (aerodontalgia) was referred pain from an occluded paranasal (probably maxillary) sinus. Not only was this pain referred to the teeth, but in an overwhelming majority of cases, it was referred to teeth which showed evidence of previous painful stimulation as by filling, trauma, caries or extraction.

This led to the hypothesis that the mechanism involved in the referral of pain in this case was one akin to spatial summation of afferent stimuli in the motor reflex and that the stimulus arising from the sinus only 'facilitated' a chronic sub-threshold stimulus from the offending tooth. Since the injury to the teeth in this study was in many cases made years previous to the observation, the authors were intrigued by this evidence of a prolonged 'central excitatory state'. Therefore, a second study (2) was set up to determine whether this observation could be repeated under conditions whereby all known factors could be controlled. Fillings and extractions were made without block anesthesia (NO was used for extractions) and after an interval of several weeks the nasal epithelium was stimulated mechanically, by a needle prick, in the region of the maxillary sinus ostium. With a single exception, pain was referred to the previously stimulated teeth whether or not any sensation was evoked from the site of the immediate stimulation.

Collateral observations made in the above studies indicated that hyperesthesia does not exist in teeth receiving dental treatment under the influence of procaine block anesthesia. This study was set up to determine whether procaine block is efficacious in removing an already established hyperesthesia.

SUBJECTS AND METHODS

The subjects reported on in this paper were the same as those used in a previous investigation (2). These had undergone dental treatment of approximately bilateral symmetry either without anesthesia, or under general nitrous oxide anesthesia. Following this treatment all subjects exhibited a tendency for occurrence of pain in the treated teeth elicited by mechanical stimulation of the ipsilateral maxillary ostium.

Since the subjects had received approximately equivalent treatment bilaterally, it was decided that the upper and lower quadrants of the dextral dentition could be used to test the efficacy of procaine anesthesia in eliminating this susceptibility to referral of pain, while the sinistral side could be left unanesthetized as a control.

In order to determine whether elevated irritability was still existent, the right maxillary ostium was mechanically stimulated, by means of a needle prick, and the occurrence of pain as reported by the subject recorded. Following this procedure, block anesthesia of the dentition in the area to which pain was referred was produced, using an injection of two per cent solution of procaine hydrochloride with epinephrine in a concentration of one part in 50,000.

The locations of the blocks were as follows:

1. For the maxillary dentition, infraorbital (anterior and middle superior alveolar branches of the maxillary division of the trigeminal nerve); infratemporal (posterior superior alveolar branches of the maxillary division of the trigeminal nerve); greater palatine and nasopalatine blocks were used.

2. For the mandibular teeth, the mandibular or inferior alveolar block was used (lingual nerve before it enters the region of the floor of the mouth and the inferior alveolar before it enters the mandibular canal at the lingula). This was supplemented by blocking the buccinator nerve.

Approximately two weeks (10 to 16 days) after production of the temporary block, both maxillary ostia were stimulated and the incidence of tooth pain recorded.

The results of stimulation of the maxillary sinus ostia are given in table 1. From table 1 it may be seen that mechanical stimulation of the maxillary sinus ostium was effective in eliciting tooth pain in dextral teeth in 100 per cent of the cases before anesthesia.

Approximately two weeks (10 to 16 days) after use of procaine block anesthesia, no referred pain to dextral teeth could be produced by ipsilateral maxillary ostium stimulation. The 'control' sinistral teeth still responded with strong pain sensation upon stimulation of the ipsilateral maxillary ostium in 13 of 14 subjects (93 per cent of the cases).

DISCUSSION

From a standpoint of practical dentistry, the observations reported present a simple method of alleviating tooth pain referred from the maxillary sinus.

Of equal importance from a fundamental standpoint are the implications of these results with respect to the neurophysiology of pain mechanisms. The authors are very fortunate in this instance in having available an example of 'pain facilitation' in which both sources of stimulation are known and capable of manipulation. Denslow and his co-workers (3, 4) have reported a closely allied condition in the case of pain perception from pressure on the spinous processes of the thoracic vertebrae. In their work a considerable advantage is reported from the presence of motor activity associated with pain transmission. This allows collection of objective evidence of neural activity which is much more

TABLE 1. EXPERIMENTAL DATA

SUBJECT		DENTAL WORK ¹		INTERVAL 1	DENTAL PAIN	ANES-THESIA	INTERVAL 2	DENTAL PAIN	INTERVAL 3
Sex	Age	Tooth	Proc	Days	Stim-1	Used	Days	Stim-2	Days
M	23	3	11	49	+	+	13	-	62
		14	1			-		+	62
M	14	4	1	53	+	+	12	-	65
		14	1			-		+	65
M	52	3	1	55	+	+	11	-	66
		14	11			-		+	66
M	28	2	1	52	+	+	11	-	63
		13	11			-		+	63
M	21	3	11	30	+	+	11	-	41
		14	1			-		-	41
M	24	4	11	50	+	+	13	-	63
		14	11			-		+	63
M	22	3	11	46	+	+	11	-	56
		13, 14	11, 1			-		+	56
M	22	3	11	59	+	+	10	-	69
		13	1			-		+	69
F	28	2, 3	11, 1	53	+	+	15	-	68
		15	1			-		+	68
F	23	3	11	50	+	+	16	-	66
		14	1			-		+	66
F	22	4	V	53	+	+	16	-	70
		13	11			-		+	70
M	30	3	11	53	+	+	14	-	67
		13, 14	1, 1			-		+	67
M	27	20	11	38	+	+	16	-	54
		30, 31	1, 1			-		+	54
F	28	3	Ext.	37	+	+	11	-	48
		13, 14, 15	Ext.			-		+	48

¹ Fillings according to Black's (1936) classification: I—occlusal surface, II—mesio or disto-occlusal surfaces; V—gingival surface; Ext.—extraction.

Interval 1. Days elapsing between original dental treatment and stimulation of maxillary sinus ostium just prior to procaine injection.

Interval 2. Days elapsing between procaine injection and final stimulation of maxillary sinus ostium.

Interval 3. Total time (days) elapsing between original dental treatment and final maxillary stimulation.

satisfactory in many respects than the purely subjective responses upon which the present paper is based. Nevertheless, the authors believe that subjective evidence of positive character, such as the dental pain used as the criterion for this study, allows a high degree of confidence in the results.

Previous work by Hardy *et al.* (5), Denslow, (3, 4), Livingston (6), Heinbecker (7) and the present authors (1, 2) has indicated that referred pain may be due to facilitation of a sensory pathway maintained at an elevated level of irritability, by stimulation of another sensory pathway in the same segment. Although this observation is fairly well established (5), there has been relatively little insight into the mechanism by which the elevated central irritability is maintained. Some workers, Sherrington (8) and Livingston (6), have suggested a 'vicious circle' concept involving afferent autonomic pathways which maintain the tissue in the region of the pain receptor in a disturbed physiologic state. Some evidence for this is found in the cases of 'phantom limb pain', causalgia and other similar conditions. Denslow reported a 'doughy consistency' of the tissue in the area of vertebral spinous processes with low pain thresholds.

In the present work, two possibilities for maintenance of elevated irritability are manifest. The simplest case would be that of a disturbed physiologic condition of the dental pulp due directly to the presence of filling materials, anatomical changes, etc. in the immediate vicinity. Another possibility is that of a disturbed circulation to the dental pulp resulting from autonomic activity in concurrence with the 'vicious circle' concept. The observations reported in the present paper indicate that the latter is the more likely explanation since reduction of the elevated irritability is effective for as long as 16 days when accomplished by a single anesthetic block of the innervation. If the elevated irritability were due to reactions continuously produced by irritating agents or structures in the dentition, one would expect that symptomology would return immediately on recovery from anesthesia.

Further, it appears from the foregoing observations that the receptor neuron itself is an important factor in maintaining an elevated central irritability. This does not discount, however, the possible importance of the autonomic innervation in maintaining a localized tissue disturbance at the site of the receptor endings.

CONCLUSIONS

1. An elevated central irritability is maintained in the sensory pathway in teeth undergoing dental treatment without local anesthesia for a long period (two months in the present paper, but previous work indicates that this may exist for several years).
2. The elevated central irritability may be reduced for an extended period by temporary procaine block anesthesia of the innervation.
3. Since referred pain to the teeth from the maxillary sinus depends upon elevated irritability of the dental sensory pathway, this procedure eliminates further dental pain of this origin either permanently or for a considerable period of time.
4. The afferent peripheral neuron itself is an important unit in the maintenance of elevated central irritability.

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RATE OF RECOVERY OF TEMPERATURE-REGULATING RESPONSES AFTER ETHER ANESTHESIA

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Received for publication February 27, 1948

All investigators who have studied the effect of anesthesia on temperature regulation of warm-blooded animals have found that anesthetics depress temperature-regulating functions. In previous work (1) it was found that morphine so disturbed temperature regulation that dogs under this anesthetic would shiver and pant simultaneously. Animals anesthetized with barbiturates, chloroform or morphine (2, 3) are unable to maintain rectal temperatures which fall one to five degrees below normal values. This inability to maintain a normal body temperature has been shown to be due to *a*) a failure of peripheral vasoconstriction in response to cold (1, 4) and *b*) a failure of the shivering mechanism to respond to a fall in rectal temperature (1).

The depressing action of anesthetics on the finer regulation of body temperature has required that physiologists studying this problem use non-anesthetized trained animals. This imposes serious limitations on many experimental methods. Where minor surgical procedures are involved, such as arterial and venous catheterization or the insertion of thermocouples in deeper structures, a possible compromise to this difficulty could consist of performing the minor surgery under a gaseous anesthetic and then allowing the animal several hours to eliminate the anesthetic after which the normal regulation of body temperature might be regained. Gaseous anesthetics are desirable due to their rapid elimination and due to the rapid recovery of the animal after discontinuing anesthesia. For these reasons a study has been made of the rate at which dogs recover their ability to regulate body temperature after thirty minutes of light ether anesthesia. The criterion for recovery of normal temperature regulation consisted of a standardized cold exposure test in which the following temperature-regulating responses were measured: *a*) ability to maintain normal body temperature, *b*) ability to produce peripheral vasoconstriction on exposure to cold and *c*) ability to shiver without an abnormal drop in rectal temperature.

EXPERIMENTAL

Medium weight short-haired dogs were used. The animals were selected and trained to lie quietly on a table beneath a wooden box cover which was joined by hinges to the table top. The cold exposure test was made in a room at 9° to 10° C. with an air velocity of 25 to 50 feet/minute and relative humidity of 50 to 75 per cent. When closed the interior of the box in which the dog was lying

was maintained at an air temperature of 34° C. by means of electrical heaters. After 30 minutes of resting in this warm environment the box heaters were turned off and the box swung open on its hinge exposing the dog suddenly to the cold environment of 10° C. During this abrupt change of environmental temperature the animal did not move but remained lying on its side in a resting position on the table top. Skin and rectal temperatures were measured throughout the test in both warm and cold environments. Exposure to cold was continued until shivering was severe as estimated by visual inspection.

The skin temperatures were measured by thermocouple junctions of 36-gauge copper and constantin wire attached to a small shave area of skin with adhesive tape $\frac{1}{4}$ " x 1". Skin temperatures were measured on the foreleg over the lower anterior surface of the tibia, the side of the thorax and the ear. As in previous work (5) it was found that the ear temperature change was the most striking evidence of peripheral vasoconstriction. A record of the changes of ear temperature and rectal temperature during cold exposure tests is given in figure 1 which includes three tests on the same dog (*dog F*): a) control (no anesthesia), b) during ether anesthesia and c) when exposure to cold, i.e., opening of the box cover, was made 153 minutes after discontinuing ether anesthesia. The onset of shivering after exposure to cold is indicated by the blocks of the diagram in the lower right of the figure.

Ether-air mixtures were prepared in two 100-liter spirometers by adding from a burette a known volume of liquid ether to a heated pressure bottle through which a measured volume of air was drawn into the spirometer. One spirometer was being filled while the air-ether mixture in the other was being breathed by the dog. A sample of the air-ether mixture was drawn from the hose leading to the tracheal cannula and analyzed for ether content. The animal was made unconscious by administration of ether by the ether cone, a procedure lasting one to two minutes. As soon as possible the cone was removed and a tracheal cannula inserted through the mouth and the prepared ether mixture was inhaled. The ether content of the gaseous mixture breathed was 10 to 11 mgm. per 100 ml. of mixture. This resulted in a blood ether concentration which varied between 100 to 120 mgm. of ether per 100 ml. of whole arterial blood. According to Beecher (6) light ether anesthesia is maintained with a blood ether concentration of 100 mgm/100 ml. of blood while Robbins (7) gives as the average anesthetic concentration of ether 115/mgm/100 ml. of blood and states that a concentration of 100 to 110 mgm/100 ml. blood is sufficient for anesthesia. The state of anesthesia observed in the experiments was estimated as being 'light' and adequate for minor surgery. Ether anesthesia was continued for 30 minutes. After discontinuing ether anesthesia the animal was allowed to recover at room temperature of 23 to 25° C. for variable lengths of time in different experiments and was placed in the warm environment at 34° C. 30 minutes before exposure to cold. In each experiment the following routine was observed:

1. *Period of anesthesia.* This period lasted 30 minutes in all experiments except those in which tests were made during anesthesia. In this latter case ether anesthesia was continued throughout all the periods. Room temperature was 23-25° C.

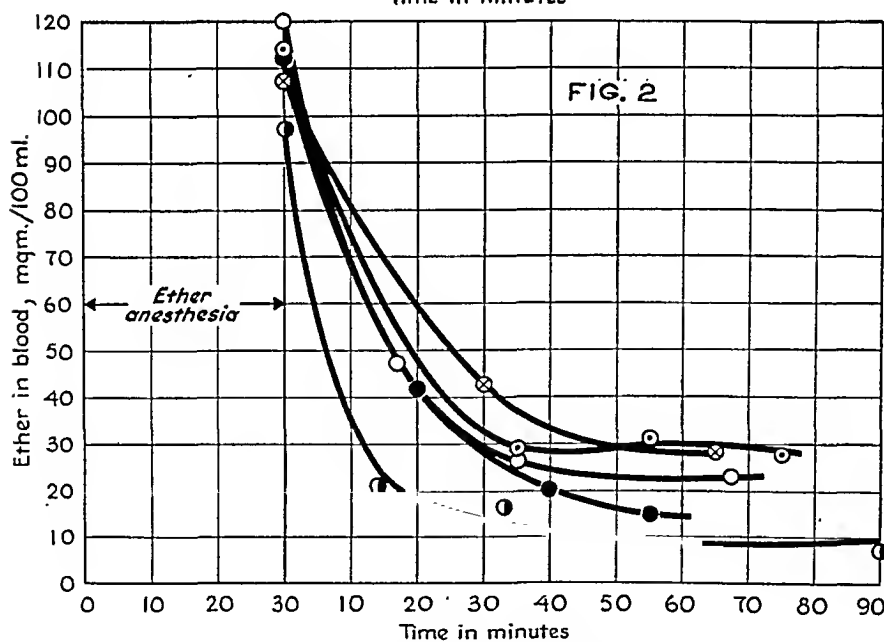
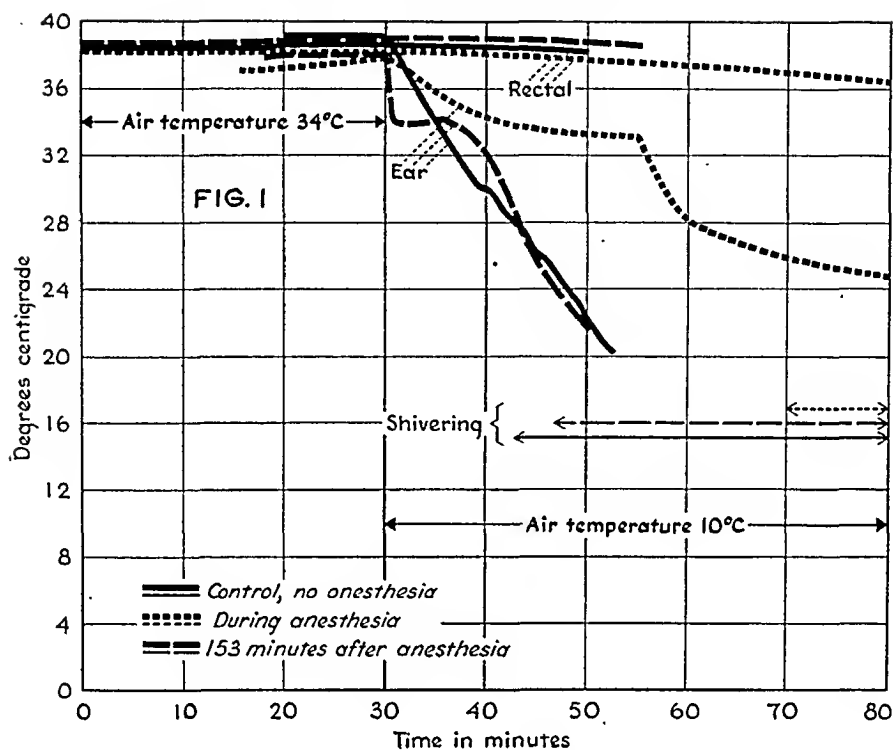


FIG. 1. RECTAL AND EAR TEMPERATURES during exposure to a warm environment of 34°C. followed by exposure to a cold environment of 10°C. Records of three experiments: a) control experiment, (no anesthesia), b) during ether anesthesia and c) exposure to cold 153 minutes after cessation of ether anesthesia.

FIG. 2. RATE OF ELIMINATION of ether from the blood after discontinuing light ether anesthesia. Five dogs were used.

2. *Recovery period.* The animal was allowed to recover from the anesthetic in an environment of 23 to 25° C. This period lasted from 0 to 150 minutes.

3. *Warming period.* The dog was rested for 30 minutes in a box at $34^{\circ} \pm 1^{\circ}$ C. in order to produce a standard thermal state.
4. *Exposure period.* The hinged box was opened and the resting animal without being disturbed was exposed to an environment of $10^{\circ} \pm 1^{\circ}$ C. The experiment terminated when shivering became violent.

Ether in air and blood was measured by the usual Nicloux oxidation method in which the ether, after distillation, is oxidized to acetic acid by a known amount of chromic acid and the excess chromic acid remaining unreduced is determined by titration. For measuring ether in blood, flasks of the Widmark (8) and Cavett (9) type were used. Recent investigators have used methyl orange as an indicator for the titration of excess chromic acid in ether and alcohol determinations. This indicator was found to give a poorly defined end point. Considerable improvement in the sharpness of the titration end point was achieved by using barium diphenylamine sulphonate in the presence of phosphoric acid, Kolthoff (10). Details of this method have been recently described (11).

RESULTS

The rate at which ether is eliminated from the blood of five dogs after 30 minutes of light ether anesthesia with blood levels of 100 to 120 mgm. ether per 100 ml. of whole blood is represented by the curves of figure 2. Ether is rapidly eliminated during the first 30 minutes after discontinuing ether anesthesia. Following this period of rapid elimination the rate decreases slowly with values of 10 to 30 mgm. ether per 100 ml. of blood being found one to two hours after anesthesia.

Data from a cold exposure test are given in figure 1. After resting for 30 minutes in a warm environment at 34° C. the animal without moving is exposed to an environment of 10° C. Only ear and rectal temperatures for one dog are given in figure 1. It will be noted that on exposure to cold there is a sudden drop of ear temperature. For the control animal the ear temperature decreased rapidly to 34° where it remained for 4 minutes and then rapidly fell to less than 20° C. The initial decrease in temperature is probably due to cooling of the skin thermocouple by the exposure to cold air where the skin temperature falls because of the cold environment. The second drop in temperature can be interpreted as being due to peripheral vasoconstriction. As an arbitrary index vasoconstriction is assumed to be in active progress when the ear temperature has reached 30° C. It will be noted in figure 1 that with a normal unanesthetized (control) animal vasoconstriction occurred after a cold stimulus of 12 minutes. With the same animal exposed to cold 153 minutes after cessation of ether anesthesia vasoconstriction occurred after a cold stimulus of 12 minutes. When the same animal was maintained under anesthesia and exposed to cold vasoconstriction (i.e., the ear temperature reached 30° C.) occurred after 28 minutes of exposure to cold.

When temperature regulatory mechanisms are depressed by anesthetics the rectal temperature of a dog at which shivering starts is lowered below control non-anesthetized values (2). Hence, one criterion of the depressant effect of

anesthesia is the rectal temperature threshold at which shivering starts. These values are given in table 1 for a) control (no anesthesia) dogs, b) dogs exposed to cold during anesthesia and c) dogs exposed to cold at varying time intervals after cessation of anesthesia. It will be noted that during anesthesia and in the first hour following cessation of anesthesia the rectal threshold values for shivering were subnormal while values two to three hours after anesthesia were normal.

It was noted in the earlier experiments on the effect of nembutal on temperature regulation (2) that the anesthetic prolongs the exposure time to cold before shivering starts. In the experiments reported here on ether anesthesia the exposure time to cold, required to produce shivering in unanesthetized, anesthetized and post-anesthesia dogs, was measured. The results are given in table 1. The 'exposure time' values are the time intervals between the opening of the box exposing the dog to the cold and the beginning of shivering. It will be observed that the exposure times were quite variable for controls as well as for the etherized animals. Except for two experiments, namely *dog A* exposed to cold 59 minutes after anesthesia and *dog C* exposed 116 minutes after anesthesia, shivering occurred during and after ether anesthesia almost as promptly as with the controls. There was a difference in the individual response of dogs. *Dog B* shivered in all experiments after 0 to 8 minutes' exposure to cold while *dog E* required 3 to 34 minutes of the cold stimulus. *Dog C* was a short-haired dog of the terrier type while *dog E* has somewhat longer and curly hair, apparently being partially of the setter type. The slow onset of shivering in *dog E* might be attributed to a difference in the length of the hair.

The test which is of most significance in evaluating the depressive action of anesthesia on physiological temperature regulation is a test of the ability to maintain rectal temperature after exposure to cold. Data from 31 cold exposure tests are given in table 2. The change in rectal temperature during a 30-minute period in the cold is given in the column ' ΔTR ' of table 2. TR is the value of the rectal temperature at the end of the rest period in the warm environment when the box was lifted and the dog was exposed to cold. The rectal temperature 30 minutes later was subtracted from TR to give ΔTR . Normal control dogs when resting in a cold environment of $10^{\circ} C.$, after changing from a warm environment of $34^{\circ} C.$, will usually have a reduction of rectal temperature varying from 0 to $0.4^{\circ} C.$ There are significant individual differences in animals as is seen in table 2. *Dog B* usually exhibited a rise in rectal temperature after exposure to cold, the increase usually being 0.1 to $0.2^{\circ} C.$ In striking contrast, the rectal temperature of *dog E* usually fell 0.3 to $0.5^{\circ} C.$ under the same conditions of testing. When the rectal temperature measurements were made during ether anesthesia the two animals tested under these conditions had rectal temperature decreases of 0.8 and $1.5^{\circ} C.$ However, two hours after ether anesthesia the decreases of rectal temperature, resulting from a 30-minute exposure to cold, were normal.

It had been found previously (5) that when dogs were exposed to cold the first response was a peripheral vasoconstriction followed secondarily by shivering. In the present experiments this procedure occurred frequently but in many cases

shivering preceded or was almost simultaneous with peripheral vasoconstriction. It was also found that the longer the experiments were continued the greater was the tendency for shivering to precede peripheral vasoconstriction. The animals

TABLE 1. THRESHOLD RECTAL TEMPERATURES AT WHICH SHIVERING STARTED AND EXPOSURE TIME TO A COLD ENVIRONMENT REQUIRED FOR INITIATION OF SHIVERING

LOG	EXPERIMENT NUMBER	RECTAL TEMPERATURE	EXPOSURE TIME	CONDITION
		°C.	min.	
A	1	39.6	2	Control, no anesthesia
	2	39.4	5	" " "
	3	39.4	8	" " "
	4	38.0	13	During anesthesia
	5	38.5	84	59 minutes after anesthesia
	6	39.2	8	60 minutes after anesthesia
	7	39.2	22	125 minutes after anesthesia
	8	39.0	13	128 minutes after anesthesia
B	1	38.6	7	Control, no anesthesia
	2	38.4	4	" " "
	3	38.3	2	" " "
	4	38.7	2	" " "
	5	37.8	0	30 minutes after anesthesia
	6	38.5	3	67 " " "
	7	38.2	0	70 " " "
	8	38.5	3	95 " " "
	9	38.4	8	125 " " "
	10	39.0	7	180 " " "
D	1	39.1	15	Control, no anesthesia
	2	38.7	11	" " "
	3	38.8	7	" " "
	4	38.1	8	78 minutes after anesthesia
	5	38.2	17	116 " " "
	6	39.1	7	120 " " "
	7	39.0	10	190 " " "
E	1	38.8	3	Control no anesthesia
	2	38.7	13	" " "
	3	38.4	34	" " "
	4	37.7	23	During anesthesia
	5	38.1	23	100 minutes after anesthesia
	6	38.4	27	153 " " "

were trained and had been tested many times in the same environment and it appeared that a state of 'conditioned shivering' was developing resembling the 'conditioned panting' to a warm environment described by Sinelnikoff (12). In other words the animals which were frequently subjected to the testing routine became conditioned and anticipated the cold by early shivering. This early

shivering apparently produced sufficient metabolic heat to delay the onset of peripheral vasoconstriction. From a study of many skin temperature records it

TABLE 2. RECTAL TEMPERATURES (TR) BEFORE EXPOSURE TO COLD AND CHANGE IN RECTAL TEMPERATURE (Δ TR) CAUSED BY 30 MINUTES EXPOSURE TO COLD. EXPOSURE TIME IN COLD BEFORE EAR TEMPERATURE REACHED 30° C.

DOG	EXPERIMENT NUMBER	TR	Δ TR	EXPOSURE TIME	CONDITION
		°C.	°C.	min.	
A	1	39.6	-0.1	—	Control no anesthesia
	2	39.4	-0.2	—	" " "
	3	39.5	-0.2	—	" " "
	4	39.0	-1.5	37	During anesthesia
	5	39.4	-0.4	8	59 minutes after anesthesia
	6	39.3	-0.3	—	60 " " "
	7	39.8 ¹	-0.7 ¹	—	125 " " "
	8	39.2	-0.2	27	127 " " "
B	1	38.5		10	Control no anesthesia
	2	38.3		—	" " "
	3	38.2	+0.1	—	" " "
	4	38.6	+0.2	—	" " "
	5	37.8	+0.1	—	30 minutes after anesthesia
	6	38.3	+0.2	—	67 " " "
	7	38.2	²	—	70 " " "
	8	38.4	+0.1	—	95 " " "
	9	38.2	³	5	125 " " "
	10	38.8	+0.3	5	180 " " "
C	1	39.4	-0.2	—	Control, no anesthesia
	2	39.0	-0.4	24	" " "
	3	38.8	-0.3	45	" " "
	4	39.2	³		78 minutes after anesthesia
	5	38.6	-0.4	25	116 " " "
	6	39.0	0.0	42	120 " " "
	7	39.2	-0.3	—	190 " " "
D	1	39.0	-0.4	15	Control, no anesthesia
	2	39.0	-0.5	14	" " "
	3	39.0	-0.5	21	" " "
	4	38.4	-0.8	28	During ether anesthesia
	5	38.8	-0.7	28	100 minutes after anesthesia
	6	38.6	-0.3	10	

¹ Warm cabinet had become overheated reaching 38° C. instead of 34° C.

² Rectal thermometer slipped from rectum.

³ Experiment terminated before 30 minutes of cooling.

has been arbitrarily decided that vasoconstriction is in active progress in the testing routine described when the falling ear temperature reached 30° C. (see fig. 1). In many tests shivering was so pronounced that the ear temperature

remained elevated for longer than the 30-minute cold period. In table 2 are given the exposure times to cold before peripheral vasoconstriction was in active progress as indicated by a fall in ear temperature of 30° C. in the cold environment of 30° C. It will be noted that peripheral vasoconstriction did occur during ether anesthesia and in the three hours following ether anesthesia. Compared with the normal controls the ether anesthesia did not prolong significantly the time required for peripheral vasoconstriction. In deep anesthesia with other anesthetics, for example, barbiturates (2), anesthesia abolishes peripheral vasoconstriction.

DISCUSSION

These results indicate that ether anesthesia in dosages necessary for light surgical anesthesia has a depressing effect on temperature-regulating responses. Neither shivering nor peripheral vasoconstriction are abolished but the fine regulation of these responses is sufficiently impaired that rectal temperature cannot be maintained. After discontinuing ether anesthesia recovery is rapid and the normal thermoregulatory state for maintenance of body temperature in the cold is reached within two to three hours after cessation of ether breathing.

SUMMARY

Medium-sized dogs were subjected to a cold exposure test for determining the thermoregulatory responses of a) shivering, b) peripheral vasoconstriction due to cold and c) maintenance of rectal temperature. The test was performed on unanesthetized dogs, dogs under anesthesia and the same animals at varying times after anesthesia. Neither shivering nor peripheral vasoconstriction are abolished by the anesthetic but fine regulation of body temperature is impaired to the degree that normal rectal temperatures cannot be maintained in the cold. The temperature regulatory responses of dogs are normal within two to three hours after cessation of ether anesthesia.

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POSTURAL CHANGES IN VITAL CAPACITY WITH DIFFERENTIAL CUFF PRESSURES AT THE BASES OF THE EXTREMITIES¹

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Received for publication February 24, 1948

The reduction in vital capacity coincident with recumbency was described by Hutchinson (1). Hamilton and Morgan (2) found that pooling of blood in the extremities produced by inflating blood pressure cuffs to diastolic level resulted in an increase in the recumbent vital capacity. Dow (3) found that in 42 normal subjects the vital capacity decreased 192 cc. on lying supine; but when pooling of blood in the four extremities was produced, the recumbent vital capacity recovered 102 cc. However, the above investigation actually produced progressive pooling of blood in the arms and legs by inflating cuffs only to diastolic level. It is the purpose of this paper to report the results obtained with blood pressure cuffs inflated to a pressure above systolic level in order to maintain the volume of blood in the extremities at a constant level.

Nine young adult male subjects were used in these studies of vital capacity in the standing and recumbent position with cuffs at the bases of the extremities inflated to diastolic pressure, above systolic pressure, and with the cuffs non-inflated. Equipment included an ordinary spirometer and a precision wet test gas meter. A pressure reservoir was utilized so that simultaneous filling of the B.P. cuffs was possible. At least 10 readings of vital capacity were taken in the various conditions for each subject.

RESULTS

Table 1 below shows that in group I there is 185 cc. mean reduction in vital capacity when a standing subject with non-inflated cuffs assumes the supine position. The latter condition is the only one in this group with a significant deviation from column 1. In column 5 the cuff pressure was raised to above systolic level just before the standing subject would recline, thus occluding arterial inflow into, as well as venous return from, the extremities. There is an insignificant 6 cc. discrepancy between columns 1 and 5, showing that the reflux of venous blood from the trunk and head did not significantly lower vital capacity in these subjects. A comparison of the first four columns reveals that cuffs maintained at diastolic level nullify the decrease in recumbent vital capacity found in the same subjects with non-inflated cuffs.

It was noted that an intense reactive hyperemia, as described by Lewis (4), follows the release of systolic pressure cuffs. In addition, bradycardia and hypotension regularly occurred with the subjects in the erect posture. Syncope was manifest in two of the five subjects studied. In the recumbent position the

¹ Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

bradycardia and hypotension did not occur although the same degree of reactive hyperemia was present. Observations on vital capacity in the erect posture with systolic cuffs cannot be repeated in close succession without obtaining fictitiously low values, due presumably to the above effect.

With this pitfall in mind, group II (table 2) was studied with reference to comparing vital capacity standing with non-inflated cuffs with vital capacity standing and supine with cuffs inflated to above systolic blood pressure. There is no sig-

TABLE 1
GROUP I¹

	STANDING WITH DEFLATED CUFFS 1	SUPINE WITH DEFLATED CUFFS 2	STANDING WITH DIASTOLIC CUFFS 3	SUPINE WITH DIASTOLIC CUFFS 4	SUPINE WITH SYSTOLIC CUFFS 5
C. S. B.	5035 \pm 16 P.E.	4837 \pm 30 P.E.	4991 \pm 8 P.E.	4889 \pm 22 P.E.	4836 \pm 33 P.E.
G. S. C.	4809 \pm 12 P.E.	4625 \pm 18 P.E.	4802 \pm 14 P.E.	4889 \pm 14 P.E.	4938 \pm 14 P.E.
K. W. C.	5302 \pm 16 P.E.	5183 \pm 15 P.E.	5289 \pm 13 P.E.	5258 \pm 11 P.E.	5266 \pm 14 P.E.
W. L. W.	5947 \pm 11 P.E.	5590 \pm 10 P.E.	6041 \pm 13 P.E.	5944 \pm 15 P.E.	6043 \pm 14 P.E.
D. J. S.	5181 \pm 9 P.E.	5112 \pm 11 P.E.	5078 \pm 13 P.E.	5155 \pm 11 P.E.	5165 \pm 9 P.E.
Mean	5254	5069	5240	5227	5249

¹ Vital capacity expressed in cc.

TABLE 2
GROUP II¹

	STANDING WITH DEFLATED CUFFS 6	STANDING WITH SYSTOLIC CUFFS 7	SUPINE WITH SYSTOLIC CUFFS 8
R. B. H.	4966 \pm 16 P.E.	5074 \pm 17 P.E.	4797 \pm 15 P.E.
C. R. H.	4014 \pm 7 P.E.	3986 \pm 13 P.E.	3776 \pm 9 P.E.
J. P. L.	5240 \pm 24 P.E.	5183 \pm 18 P.E.	4826 \pm 15 P.E.
H. L. H.	4736 \pm 17 P.E.	4788 \pm 9 P.E.	4595 \pm 12 P.E.
Mean	4746	4757	4498

¹ Vital capacity expressed in cc.

nificant difference in the vital capacities of subjects standing with non-inflated cuffs as opposed to standing with cuffs at above systolic pressure.

The subjects in group II had the cuffs raised to above systolic blood pressure after reclining for 3-5 minutes (in contrast to group I whose cuffs were inflated before the subjects reclined). Consequently column 8 displays a significant 248 cc. reduction in vital capacity when compared to column 6, indicating that the pooling of blood in the lungs of a recumbent individual will in less than 3-5 minutes result in a decreased vital capacity.

CONCLUSION

1. The vital capacities were determined in nine adult male subjects in the standing and supine positions with cuffs placed at the bases of the arms and legs

inflated to diastolic pressure, above systolic pressure, and non-inflated. When the cuffs were inflated to a level above systolic blood pressure before the standing subjects would recline, the reflux of venous blood from the trunk and head did not lower vital capacity.

2. There was no difference between the vital capacity of subjects standing with the cuffs inflated above systolic pressure and the vital capacity of these same subjects standing with non-inflated cuffs.

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ALVEOLAR GAS CHANGES DURING BREATH HOLDING¹

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Received for publication January 5, 1948

Although there are in the literature numerous studies of the maximum time that the breath can be held under various conditions (1), there is little information available regarding the changes in alveolar gas composition during breath holding. The composition of the alveolar air at the end of the involuntary apnoea (not the maximum voluntary apnoea) produced by two minutes of hyperventilation has been measured by Douglas and Haldane (2) with various concentrations of oxygen in the air inspired during the period of forced breathing. The same authors (3) as well as Hill and Flack (4, 5) have compared the alveolar compositions existing at the end of maximum breath holding when air and when pure oxygen were previously breathed. Ferris *et al.* (6) have measured the $p\text{CO}_2$ and $p\text{O}_2$ of the arterial blood at the 'breaking point' and recognize these factors as interrelated stimuli for termination of breath holding.

It is the purpose here to present data showing the alveolar gas exchange during periods of breath holding following the breathing of various inspired oxygen tensions and to consider the factors determining the maximum duration of breath holding.

METHODS

In all cases the breath was held at the end of a normal expiration in the sitting position. The same eight trained subjects² were used in all experiments. The breath was held as long as possible and the expiratory reserve was then vigorously blown through a large stopcock into a rubber tube two meters long and 2.5 centimeters in diameter. As soon as the expiration was complete the stopcock was closed to trap the sample of alveolar air in the tube. A small side arm just below the stopcock delivered the sample to the oxygen and carbon dioxide meters (7) where it was immediately analyzed.

Low oxygen tensions in the inspired air were obtained in a high altitude chamber by going in succession to simulated altitudes of 8, 12, 16 and 18 thousand feet. The subjects, who wore ear oximeters, remained at each altitude for about twenty minutes and delivered two samples which were analyzed at that altitude. Upon return to ground level the breath-holding test was repeated.

Oxygen tensions higher than normal were obtained by breathing 32 per cent

¹ Work done under contract recommended by Committee on Medical Research between the Office of Scientific Research and Development and the University of Rochester, and under contract with the Air Materiel Command, Wright Field.

² We wish to acknowledge the fine cooperation of the members of Civilian Public Service Unit No. 115-R who served as subjects and technicians during these experiments.

and 45 per cent oxygen in nitrogen and 100 per cent oxygen from large spirometers at ground level for ten minutes before the breath was held. Each test was repeated twice or until reliable checks were obtained upon repeated trials.

RESULTS

At ground level, and particularly at the higher altitudes, there was no hesitation on the part of the subjects in deciding exactly when further apnoea was impossible. On the other hand, at the two highest oxygen concentrations it was often rather difficult to decide when the real breaking point had been reached. While holding the breath at these high oxygen tensions, the subjects experienced involuntary contractions of the respiratory muscles which became increasingly violent and rapid. At first they involved the diaphragm and later the intercostals and accessory breathing muscles. Simultaneously the subject experienced sensations of intense warmth with flushing of the skin and sweating, particularly of the forehead and of the palms of the hand. Even so, the first minute of apnoea on pure oxygen frequently seemed more uncomfortable than the last minute.

At the 18,000-foot altitude all subjects came very close to fainting. Some actually lost postural control momentarily with involuntary swaying of the body or head. Two men, however, were able to carry out the breath-holding maneuver successfully at 20,000 feet.

A summary of the average breath-holding times for eight subjects at 10 different tensions of inspired oxygen is given in table 1. Data are also given for ear oximeter readings at the breaking point, mean R.Q. during the period of apnoea and terminal values of alveolar $p\text{CO}_2$ and $p\text{O}_2$. In general the higher the O_2 tension, the higher the CO_2 at the breaking point and the longer the period of voluntary apnoea. The observed arterial saturations are all slightly lower than the values calculated from the alveolar gas tensions, presumably because the oximeter falls suddenly at the time of forcible expiration, as if from some circulatory disturbance, and we recorded only the lowest values.

The data relating to composition of the alveolar gas are graphically depicted in figure 1 by plotting them on a $p\text{CO}_2$ - $p\text{O}_2$ diagram (8). The numbered circles indicate the average breaking points in the various experiments and form the basis for constructing the breaking point curve. This curve also adequately fits the breaking points determined from analysis of arterial blood by Ferris *et al.* (6). It is also similar in shape to the curve of Douglas and Haldane (2) showing the points at which involuntary apnoea following hyperventilation ceases, but is located higher on the diagram. The starting point for each test is indicated by the appropriate $p\text{CO}_2$ - $p\text{O}_2$ value on the normal alveolar gas curve (constructed from our own data). The dotted lines leading from the starting points to the breaking points indicate the presumed time course of the alveolar gas changes during the breath-holding period. (The actual time course is known in only three subjects and will be discussed below.) Except for the lower tensions of inspired oxygen, the slopes of these lines become less with time, because the O_2 uptake from the lungs remains relatively constant while the CO_2 output di-

TABLE 1. SUMMARY OF ALVEOLAR GAS TENSIONS AND OTHER DATA IN BREATH HOLDING (B.H.) EXPERIMENTS

(1) Experiment no..	1	2	3	4	5	6 ²	7	8	9	10
(2) No. of subjects..	8	8	8	8	8	8	8	8	6	7
(3) No. of expts....	12	16	16	16	16	16	22	15	13	14
(4) B-47 in mm. Hg.	332	365	436	517	697	697	702	700	691	694
(5) Altitude in thousands of feet..	18	16	12	8	0.5	0.5	0.5	0.5	0.5	0.5
(6) Inspired pO ₂	69	76	91	108	146	146	147	227	317	694
(7) Inspired O ₂ %...	Air	Air	Air	Air	Air	Air	Air	32.4	45.9	100
(8) Alveolar pO ₂ before breath hold.....	36	41	50	62	104	104	104	187 ¹	277 ¹	656
(9) Alveolar pCO ₂ before breath hold.....	30	33	37	37	38	38	38	38	38	38
(10) Alveolar pN ₂ before breath hold ⁵	266	291	349	418	555	555	560	475	376	0
(11) Alveolar pO ₂ after breath hold.....	28.0	31.1	36.1	44.2	62.7	45.9	52.1	72.5	157	628 ³
Std. dev. ±.....	1.0	2.9	1.4	3.0	9.9	6.7	9.0	16.0	34.2	3.9
(12) Alveolar pCO ₂ after breath hold.....	38.6	42.3	45.6	46.9	50.1	48.3	50.2	57.5	62.6	66.3
Std. dev. ±.....	2.8	2.6	1.9	1.2	1.3	3.1	1.7	4.4	5.6	3.9
(13) Alveolar pN ₂ after breath hold ⁵	265	292	354	426	584	603	600	570	471	0
(14) Duration of B.H. in seconds....	23	25	27	30	35	59	50	92	106	153
Std. dev. ±.....	6.4	3.7	6.4	8.7	4.4	18.3	17.1	28.9	38.9	53.5
(15) HbO ₂ % by oximeter.....	48	51	61	71	87	70				
(16) HbO ₂ % by calculation.....	55	58	67	77	90	78	86	92		
(17) Lung volume after B.H., liters.....	3.0	3.0	2.97	2.97	2.85	2.76	2.80	2.50	2.40	2.42 ⁴
(18) O ₂ intake rate during B.H., cc/min.....	73	84	109	143	264	220	230	289	300	
(19) Average R.Q. during breath hold.....	1.07	.93	.57	.48	.22	.10	.16	.08	.08	

¹ Assumed 40 mm. Hg less than inspired pO₂ in row 6.² Observations made immediately after hyperventilation caused by previous 'ascent' to altitude.³ Calculated as row 4 minus row 12. Measurements on the O₂ meter are very inaccurate at this level.⁴ Calculated by equation 6 assuming Q = 0.1.⁵ pN and p'N were calculated by subtracting the CO₂ and O₂ tensions from B-47 in row 4.

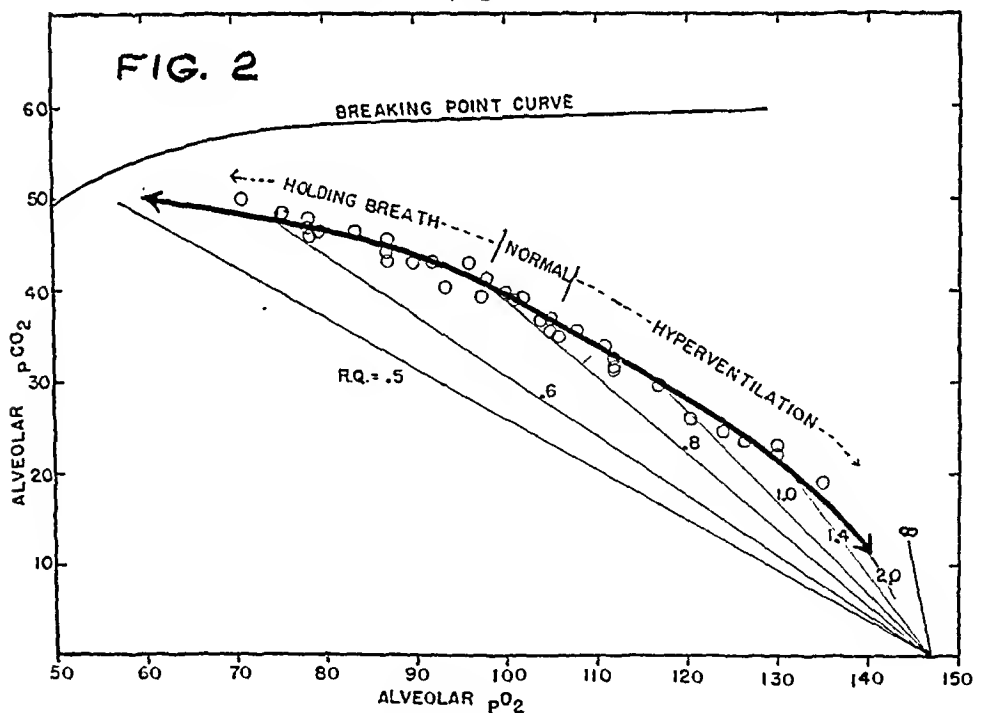
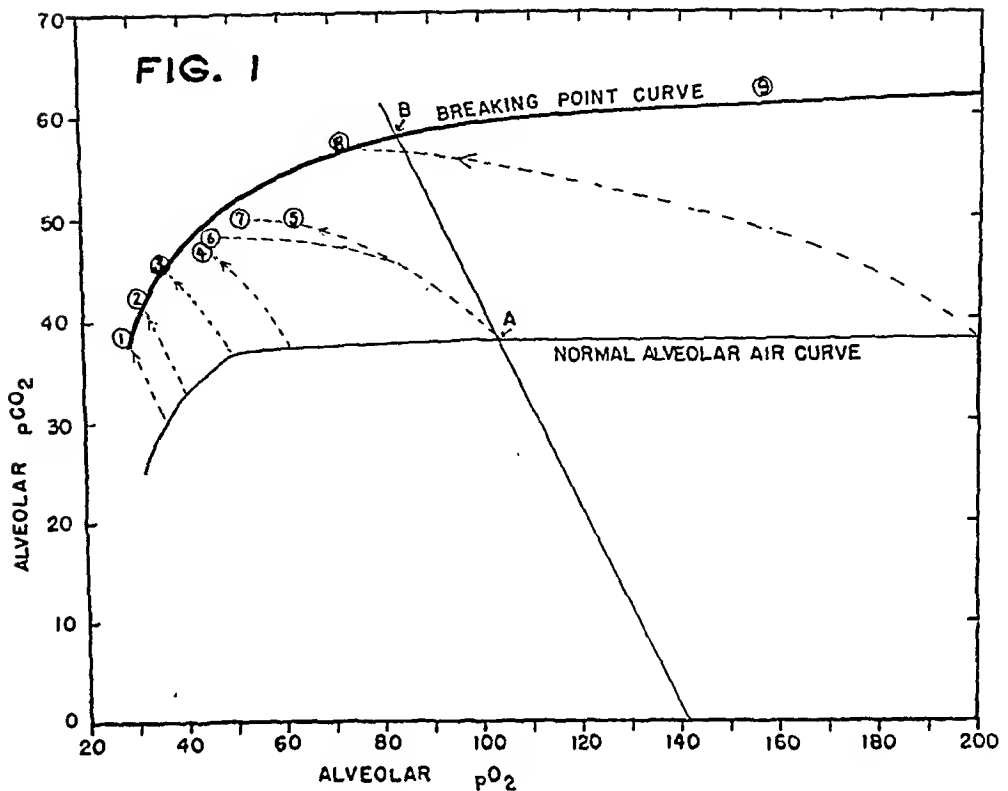


FIG. 1. THE PRESUMED COURSE OF CHANGE in alveolar gas composition during breath holding under various conditions.

FIG. 2. THE ALVEOLAR PATHWAY during breath holding and during hyperventilation.

minishes. This is explained by the fact that although an ample gradient for transfer of O_2 from lungs to blood exists throughout the breath-holding period, the gradient for transfer of CO_2 in the opposite direction becomes progressively less favorable owing to the ever increasing pCO_2 in the lungs.

At the lower inspired O_2 tensions, however, the slopes of these dotted lines are steeper in general and do not fall off much with time. At these O_2 tensions the alveolar diffusion gradient for oxygen is so much less than normal that the O_2 uptake from the lungs is lower and more nearly equal to the CO_2 transfer. Another manifestation of these processes is that the rate of change of lung volume during breath holding is greater the higher the pO_2 of the inspired air (9).

The dotted lines in figure 1 represent the presumed time course of the alveolar air composition while breathing air at different altitudes. The time course depends, however, not only upon the initial tension of oxygen but also on the percentage of oxygen. Point *A* in figure 1 represents the alveolar air at ground level, breathing air. The same tensions of oxygen and carbon dioxide can be obtained by breathing pure oxygen at an altitude of approximately 34,000 feet. In this case when the breath is held, the composition of the alveolar air must remain somewhere on the line *AB*, this being the diagonal for an R.Q. of 1.0 where $pCO_2 + pO_2 = 142$ (8). The carbon dioxide tension at point *B* is far higher than at point *A*, breathing air. One of our subjects carried out this experiment and found that the pCO_2 at the breaking point was 48 mm breathing air at ground level but was 62.5 mm at 34,000 feet breathing pure oxygen. Likewise the pO_2 was higher and the period of voluntary apnoea longer in the latter case. This experiment confirms, therefore, the theoretical expectations. If the lungs contain pure O_2 , a passage of O_2 into the blood causes a decrease in volume but no change in O_2 tension. In the presence of a large amount of N_2 , however, a decrease in volume due to diffusion of O_2 results chiefly in increasing the pN_2 so that the O_2 tension becomes much less than when pure O_2 is breathed. This would tend to decrease the amount of CO_2 which can be tolerated and should shorten the breath-holding time. At the same initial oxygen tension, therefore, the greater the percentage of oxygen the less the decrease in pO_2 for the same O_2 consumption. As we have shown elsewhere (8) the R.Q. diagonals deviate less and less from the R.Q. = 1 line as the O_2 percentage increases until at 100 per cent O_2 they all coincide. The breath-holding experiment referred to is a manifestation of this phenomenon. The observation of Brown (10) that the breath can be held longer at 30,000 feet on pure O_2 than at ground level on air is also consistent with this, but the results of Rodbard (1) who found that breath-holding times on oxygen at 28,000 feet were less than at ground level on air cannot be explained on this basis.

The changes in the R.Q. and the sequence of the changes in alveolar composition during breath holding are shown in figure 2, which represents data on three subjects breathing air. Starting with an alveolar air represented by the region of the curve marked 'normal', the breath was held for various periods of time, after which the alveolar air was exhaled and analyzed. The alveolar point moves up the 'breath-holding curve' and gradually approaches the breaking point line.

In the other direction the line can be extended from the normal region to lower $p\text{CO}_2$ values by voluntary hyperventilation for different periods at a high rate. In this way the alveolar air changes so as to approach the point on the axis of abscissae from which the R.Q. lines radiate (8). This point represents the composition of the inspired air and can be reached only by an infinite ventilation rate. According to the chart, the R.Q. increases during hyperventilation. Thus the R.Q. is 1.4 where the hyperventilation curve intersects the 1.4 diagonal. When the breath is held the R.Q. becomes progressively less. In this case, however, the numerical magnitude of the R.Q. is not given by the diagonal R.Q. lines, because the original inspired air point can no longer apply when the glottis is closed, but rather by the slope (approximately) of the breath-holding curve. At the beginning of apnoea the slope of this curve is such that $\frac{\Delta p\text{CO}_2}{\Delta p\text{O}_2} = 0.5$. Since the $p\text{N}_2$ is fairly large in this case the total volume of the lung does not change much so that without serious error $\frac{\Delta p\text{CO}_2}{\Delta p\text{O}_2} = \text{R.Q.}$ The mean R.Q. during the

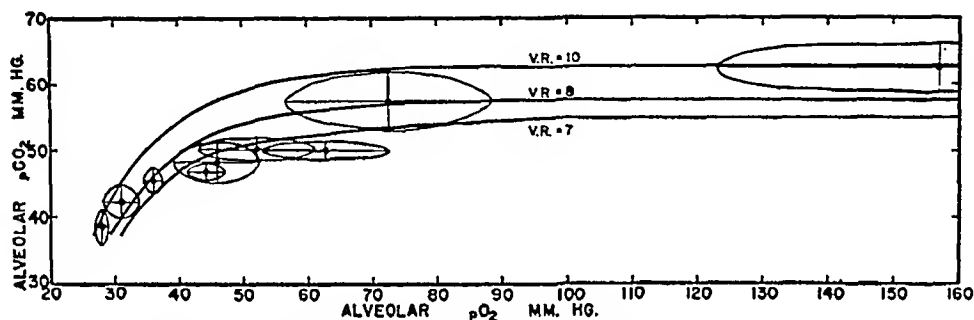


FIG. 3. THEORETICAL BREAKING POINT CURVES constructed as described in the text. The points are plotted from the experimental data in table 1. The standard deviation of each point is indicated by an ellipse.

whole period of apnoea is of course much less. Where the breath-holding curve crosses the R.Q. = 0.5 diagonal, the slope of the curve actually indicates an R.Q. of 0.15. The correct method of calculating the R.Q. from the slope of the breath-holding curve will be given below. (See equations 6 and 7.)

DISCUSSION

For a more quantitative discussion of these results the following symbols will be used:

Let $p\text{O}$, $p\text{C}$ and $p\text{N}$ be alveolar tensions of O_2 , CO_2 and N_2 before breath holding.

$p'\text{O}$, $p'\text{C}$ and $p'\text{N}$ = similar tensions at the breaking point

FC and $\text{F}'\text{C}$ = the fraction of CO_2 in the lungs before breath holding and at the breaking point, respectively

V and V' = lung volumes in cc. BTPS before and after breath holding.

x and y = cc. of O_2 lost and CO_2 added, respectively, during breath holding at BTPS.

B = ambient pressure in mm Hg

t = breath-holding time in minutes

X_0 = cc. O_2 diffusing out of lung per min. at S.T.P.

Q = normal alveolar R.Q. preceding breath holding

Q' = mean alveolar respiratory quotient during time t

W = an unknown virtual volume of body water

α = slope of the CO_2 dissociation curve for W. $\alpha = \frac{3500 \text{ cc. } CO_2 \text{ (S.T.P.)}}{\text{liter/atm.}}$

Calculation of alveolar oxygen consumption during breath holding

$$x = \frac{pOV - p'OV'}{B - 47} \quad (1)$$

$$\begin{aligned} X_0 &= \frac{pOV - p'OV'}{(B - 47)t} \times \frac{273}{310} \times \frac{B - 47}{760} \\ &= \frac{pOV - p'OV'}{863t} \end{aligned} \quad (2)$$

The amount of nitrogen in the lung may be assumed to remain constant during breath holding,³ so

$$p'NV' = pNV$$

and

$$V' = \frac{pN}{p'N} V \quad (3)$$

Substituting (3) in (2) gives

$$X_0 = \frac{\left(pO - p'O \frac{pN}{p'N}\right) V}{863t} \quad (4)$$

The average rates of oxygen consumption have been calculated for the breath-holding periods according to equation 4 by assuming an initial lung volume of 3000 cc. and the results are given in table 1. No value is given for experiment 10, because no nitrogen was present and equation 4 does not apply. Equation 2 could have been used in this case had the change in lung volume been known. It is apparent that the rate of oxygen consumption decreases from a normal value of 300 cc/min. during 106 seconds of breath holding at high oxygen tensions to 73 cc/min. for the 23-second period at 18,000 feet. A decrease in the intake of O_2 from the lungs indicates either that the metabolic rate is decreasing or that the O_2 reserves in the body (chiefly blood HbO_2) are being drawn upon. That the latter is more probable is indicated by the following argument.

³ We have calculated that in the experiments which involve the greatest change in pN_2 (columns 8 and 9 of table 1) the amount of nitrogen taken out of the lung by the blood would be less than 1 per cent of the total nitrogen present.

At 18,000 feet the breath was held for 23 seconds and during that time only 28 cc. of O_2 were removed from the lungs instead of the normal value of about 120 cc. At the same time, however, the arterial saturation fell from a probable value of 70 per cent to an observed value (oximeter) of 48 per cent. If the average fall in all the blood (including venous) were only half as great, at least 10 per cent of the oxygen capacity of the blood or about 100 cc. could have been consumed in the tissues. It may be supposed, therefore, that the O_2 actually used by the tissues does not decrease during the period of breath holding even at anoxic altitudes.

Calculation of the mean alveolar R.Q. during breath holding

$$y = \frac{p'CV' - pCV}{B - 47} \quad (5)$$

$$Q' = \frac{y}{x} = \frac{p'CV' - pCV}{pOV - p'OV'} \quad (6)$$

Substituting (3) in (6)

$$Q' = \frac{p'C \frac{pN}{p'N} - pC}{pO - p'O \frac{pN}{p'N}} \quad (7)$$

Equation 7 has been used to calculate the mean R.Q. during the breath-holding period for all experiments where nitrogen was present. The results are recorded in table 1. At 18,000 and at 16,000 feet the average R.Q. for the period of apnoea is 1.07 and 0.93, respectively, but at ground level on air the value is 0.1 to 0.22 and when extra O_2 is added, values as low as 0.05 result. When the R.Q. is less than unity, there is a decrease in lung volume during the breath-holding period. The lung volume at the end of the breath-holding period may be computed by equation 3. Results of this calculation, assuming an initial lung volume of 3.0 liters, are recorded in table 1. When the breath is held on pure O_2 , equations 3 and 7 do not apply, but a relationship between lung volume change and R.Q. is given by equation 6. The final lung volume in experiment 10 has been estimated by the use of this equation, assuming an R.Q. of 0.1 and an initial lung volume of 3.0 liters.

The lung volume is not significantly lowered during breath holding on air at high altitudes, because the R.Q. is about unity, but at ground level and at high oxygen tensions the volume decreases from an initial value of 3.0 liters to a final value of 2.4 liters. Since the breath was held at the end of a normal expiration, this means that the final lung volume is 600 cc. less than the relaxation volume and that some expiratory effort is required to avoid the development of negative pressure within the lungs (11). Breath holding on pure oxygen at high altitudes involves an even greater decrease in volume. We have not investigated this matter thoroughly, but one subject who held his breath on oxygen at 34,000 feet was unable, at the breaking point, to expire enough gas to give a sample for

analysis. It is probable that under such conditions, a decrease to the residual volume might be the decisive factor terminating breath holding.

The high R.Q. values observed during breath holding at anoxic altitudes are the result of the decreased oxygen consumption that occurs under these conditions as described above. The low R.Q. values obtained at ground level where the oxygen consumption remained normal are due to retention of CO₂ in the blood and other tissues, the rising pCO₂ in the lungs creating a less favorable gradient for CO₂ transfer. The amount of CO₂ retained depends on the relative volumes involved of lung air and tissue water, on the slope of the CO₂ dissociation curve of the tissue water and on the length of time available for equilibration.

The total CO₂ (S.T.P.) formed during a period of breath holding is given by

$$(F'CV' - FCV) \left(\frac{B - 47}{760} \times \frac{273}{310} \right) + (F'C - FC)\alpha W \quad (8)$$

If the alveolar O₂ consumption remains the same as before breath holding.

$$\frac{(F'CV' - FCV) \frac{(B - 47)}{(863)}}{(F'CV' - FCV) \frac{(B - 47)}{(863)} + (F'C - FC)\alpha W} = \frac{Q'}{Q} \quad (9)$$

Multiplying by $\frac{B - 47}{B - 47}$ to convert 'F' to 'p' gives

$$\frac{(p'CV' - pCV) \frac{(B - 47)}{(863)}}{(p'CV' - pCV) \frac{(B - 47)}{(863)} + (p'C - pC)\alpha W} = \frac{Q'}{Q} \quad (10)$$

If values from table 1 for experiment 5, in which the breath was held on air at ground level for 35 seconds, are substituted in this equation, W is found to be 1.57 liters. For experiment 9, in which the breath was held 106 seconds on a higher oxygen mixture, W comes out to be 3.08 liters. The value of 3500 for α is the slope of the CO₂ dissociation curve for blood at 45 mm Hg from Field *et al.* (12). Some justification for using this value for the tissues is found in a paper by Adolph *et al.* (13), who found that for a given rise in CO₂ tension, 3-18 per cent of the CO₂ was absorbed by the blood and 82-97 per cent or about nine times as much, on the average, by the tissues. Since the total volume of body water is about nine times the blood volume, the slope of the CO₂ dissociation curves for blood and tissues must be about equal. If α is taken as only half that of blood, as indicated by the experiments of Shaw (14), the values of W will be doubled, but at any rate these calculations indicate that the CO₂ formed during breath holding equilibrates virtually with only a small fraction of the total 40 or 45 liters of body water. The blood volume alone is ample to account for it.

Actually, of course, the total blood volume is not equilibrated nor is any other tissue. The blood leaving the lungs has a progressively higher pCO₂ as breath holding continues, and the tissues with the best circulation will be most nearly

equilibrated. The more actively metabolizing tissues such as brain and kidney will not only produce more of the CO_2 formed during breath holding but will also retain a larger amount, because of their more rapid circulation. Some tissues may be so slowly circulated that during the shorter periods of breath holding they are not affected at all. For a tissue to retain CO_2 during the breath-holding period the duration must be at least as long as the circulation time from the lungs to that tissue. The steady decrease with time in the value $\frac{\Delta pC}{\Delta pO}$ that occurs when

the oxygen uptake from the lungs remains constant during breath holding is due, therefore, to retention of CO_2 in progressively greater fractions of the body water. Presumably if the breath were held long enough to stop all O_2 consumption and CO_2 production, and if the circulation continued long enough, the $p\text{CO}_2$ in the lungs would slowly fall, as the poorly circulated areas of low metabolic rate gradually took up from the lungs their share of the CO_2 formed during the apnoea. A slight fall of alveolar $p\text{CO}_2$ has in fact been observed in obstructive asphyxia by Thompson and Birnbaum (15) and by Grodins *et al.* (16), but in similar experiments on dogs in this laboratory (17) the heart apparently stopped before the fall could be recorded.

The theoretical breaking point. Gray (18) has shown how various stimuli and inhibitions to breathing and their inter-relationships may be expressed in a quantitative fashion. The success with which Gray applied his approach to various respiratory problems suggests the application of his methods to the phenomenon of breath holding.

Breath holding is an example of the voluntary inhibition of an ordinarily involuntary process. It seems reasonable to assume that if one could express inhibition in quantitative units, maximum voluntary inhibition would be a constant value independent of the nature of the opposing stimuli. The breaking point in breath holding might then be considered as the point at which the stimulus to breathe becomes equal to the maximum voluntary inhibition.

The principal stimuli acting during breath holding are probably the $p\text{CO}_2$, the H ion concentration, and the $p\text{O}_2$ of the arterial blood. Gray has shown that the partial effects of each of these stimuli may be computed independently and that the net total stimulus is the algebraic sum of all three. The *chemical ventilation equation* describing this relationship is:

$$V.R. = 0.22 H + 0.262 p\text{CO}_2 - 16 + \frac{105}{10^{0.038 p\text{O}_2}}$$

where *V.R.* is the ventilation ratio or the ratio of the observed to the normal alveolar ventilation. If the bicarbonate capacity is normal, *H* and $p\text{CO}_2$ are so related that the above equation is simplified to:

$$V.R. = 0.4 p\text{CO}_2 - 15 + \frac{105}{10^{0.038 p\text{O}_2}}$$

This equation applies exactly to steady state conditions only. Since no equation of this sort has yet been derived to describe the particular unsteady state

that exists during breath holding, the above equation has been used to estimate the stimulus strength existing at the breaking point for each set of data in table 1. The results of these calculations are shown in table 2. The different values obtained for the total stimulus at the breaking point are hardly constant as would be demanded by an hypothesis of a constant maximum voluntary inhibition. This discrepancy may be due to the inaccuracy previously pointed out of applying a steady state equation to an unsteady state, to a less close approach to equilibrium between alveolar air and arterial blood during breath holding than during normal breathing, (although the close correspondence between our breath-holding curve based on alveolar air values and a similar curve based on arterial

TABLE 2. THE THEORETICAL VENTILATION STIMULUS EXISTING AT THE BREAKING POINT OF BREATH HOLDING

pCO ₂	pO ₂	V.R. _{H,pCO₂}	V.R. _{pO₂}	V.R. _{Total}
38.6	28.0	0.4	9.1	9.5
42.3	31.1	1.9	6.9	8.8
45.6	36.1	3.3	4.3	7.8
46.9	44.2	3.8	2.2	6.0
50.1	62.7	5.0	0.4	5.4
48.3	45.9	4.3	1.9	6.2
50.2	52.1	5.1	1.1	6.2
57.5	72.5	8.0	0.2	8.2
62.6	157.	10.0	0	10.0
66.3	527.	11.5	0	11.5
Mean.....				8.0

Explanation of column headings:

pCO₂ and pO₂ refer to the alveolar gas tensions in mm. Hg measured at the breaking point.

V.R._{H,pCO₂} = $0.4p\text{CO}_2 - 15$, and is the partial ventilation stimulus due to the H ion, pCO₂ complex.

V.R._{pO₂} = $\frac{105}{10^{0.038p\text{O}_2}}$ and is the partial ventilation stimulus due to pO₂.

V.R._{Total} = V.R._{H,pCO₂} + V.R._{pO₂} and is the total stimulus to breathe at the breaking point. According to the proposed hypothesis, it is also equal to the maximum voluntary inhibition.

blood values (6) would seem to minimize this) and possibly to the action of stimuli such as reflexes from the lungs and formation of lactic acid which are not taken into account in the equation. The calculated partial stimulus effects of pCO₂ and of pO₂ in each case are probably relatively correct and indicate that the stimulus at the breaking point in the various experiments of table 1 may be a) completely due to high pCO₂, b) almost completely due to low pO₂ or c) due to various combinations of hypercapnic and hypoxic stimuli. The mean value for the total stimulus at the breaking point (V.R. = 8) has been used as a basis for constructing a theoretical curve for breath holding. By the method indicated in table 3 various pCO₂, pO₂ combinations that should produce a total V.R. = 8 have been calculated. The theoretical breaking point curve for breath holding

has been plotted from these calculated $p\text{CO}_2$, $p\text{O}_2$ combinations on figure 3 (curve labeled V.R. = 8) along with the original experimentally determined points. Two other similarly constructed curves assuming constant stimuli of V.R. = 7 and V.R. = 10, respectively, are shown on the same graph for comparison. These curves are of approximately the proper shape, and except at the higher CO_2 tensions, the fit of the curve for V.R. = 8 is hardly worse than the scatter of the original data, which is indicated by the ellipses of standard deviation surrounding each point.

Although the hypothesis that the breaking point of breath holding occurs when the total stimulus to breathe becomes equal in value to a constant maximum voluntary inhibition is not proven by the above treatment, the results are gratifying enough to suggest further work. For example, the curve for the breaking point should be displaced downwards and to the right in persons acclimatized to

TABLE 3. CALCULATED $p\text{CO}_2$, $p\text{O}_2$ VALUES THAT WILL GIVE A TOTAL VENTILATION RATIO EQUAL TO 8

V.R. \cdot H, $p\text{CO}_2$	V.R. \cdot $p\text{O}_2$	$p\text{CO}_2$	$p\text{O}_2$
8	0	57.5	100
7	1	55.0	53.2
6	2	52.5	45.2
5	3	50.0	40.5
4	4	47.5	37.4
3	5	45.0	34.7
2	6	42.5	32.6
1	7	40.0	31.0
0	8	37.5	29.3

Explanation of column headings:

V.R. \cdot H, $p\text{CO}_2$ refers to the partial stimulus effect assumed due to the H ion, $p\text{CO}_2$ complex.

V.R. \cdot $p\text{O}_2$ = 8 - V.R. \cdot H, $p\text{CO}_2$

$p\text{CO}_2$ was calculated from the equation, V.R. \cdot H, $p\text{CO}_2$ = $0.4 p\text{CO}_2 - 15$

$p\text{O}_2$ was calculated from the equation V.R. \cdot $p\text{O}_2$ = $\frac{105}{10^{0.038p\text{O}_2}}$

altitude because, as Gray has pointed out, the ventilation of such individuals has increased sensitivity to CO_2 . Exercise, either active or passive, should also similarly displace the curve because a strong reflex stimulus to breathe would be added to the H, $p\text{CO}_2$, $p\text{O}_2$ combination. The maximum voluntary inhibition estimated above and expressed as V.R. = 8 is close to the maximum ventilation rate attained during active muscular exercise. If a person exercised at a rate such that his ventilation equalled the maximum voluntary inhibition, then he should not be able to stop breathing at all. His breath-holding time would be zero.

SUMMARY

1. Breath holding was investigated in eight subjects at inspired oxygen tensions varying from 69 to 694 mm Hg. The time, HbO₂ per cent, CO_2 and O_2 alveolar tensions were recorded at the breaking point.

2. At high O_2 tensions the breath-holding time was long, the R.Q. was 0.05 to 0.1 due to retention of CO_2 and the rate of O_2 consumption during apnoea was normal.

3. At low O_2 tensions (18,000 feet) the breath-holding time was short, the rate of O_2 consumption only about one-fourth the normal, so that the R.Q. was about 1.0.

4. When R.Q. is less than 1.0 the lung volume tends to decrease. If N_2 is present this results chiefly in increasing pN_2 so that pO_2 decreases rapidly. When N_2 is absent the decrease in volume chiefly prevents the pO_2 from falling, so that the pCO_2 is much higher at the breaking point for the same initial alveolar pO_2 .

5. Theoretical treatment of the data indicates that the breaking point of breath holding occurs when the sum of the separate partial stimuli from low pO_2 and from high pCO_2 gives a total stimulus sufficient to produce an alveolar ventilation about eight times the normal.

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METABOLIC CHANGES OF BLOOD AND TISSUE GASES DURING ASPHYXIA¹

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Received for publication January 19, 1948

Recently several investigators (1-4) have questioned the alleged benefits of 5 to 7 per cent CO₂ in oxygen as a resuscitating agent for cases of obstructive asphyxia. The present study was undertaken, first, in order to confirm evidence concerning the acid-base balance of the blood during obstructive asphyxia, and thereby to aid in the evaluation of the CO₂-O₂ mixture as a resuscitating agent in such cases. Secondly, we have expanded that evidence to include a simultaneous study of blood lactic acid levels, (a measure of the degree of metabolic acidosis during asphyxia), and other pertinent variables to be indicated. Thirdly, we have made a preliminary study of the metabolic activity of muscle tissue during obstructive asphyxia. Efforts have been made to correlate it with simultaneous acid-base variations in the blood.

METHODS

The study of 10 healthy dogs, anesthetized by intraperitoneal injections of Dial² to surgical anesthesia, is reported here. The trachea was cannulated, and each dog was asphyxiated by rebreathing from a small spirometer a quantity of room air approximately equal to four times its tidal volume. Respiratory excursions throughout the asphyxiating procedure were recorded on a moving drum by a writer attached to the spirometer bell. A 5 cc. alveolar air sample was withdrawn from the rebreathing tube into an airtight syringe every one or two minutes during the asphyxiating procedure. Just before each sample was drawn, the chest was forcibly compressed, and held in this position during the sampling. The syringes were inverted in tubes of mercury, and so sealed until analyzed for CO₂ content within 24 hours by the Fry (5) gas analyzer.

Every one or two minutes during the asphyxiating procedure a 5-6 cc. sample of arterial blood was withdrawn from the carotid artery through a blunted needle into a syringe, the dead space of which had been filled with 6.5 percent NaF. The exact amount of fluoride in each syringe was determined, and this dilution factor considered when calculating blood concentration from the analytical figures. Approximately 6 mgm. of fluoride per cc. of blood was found to be sufficient to prevent clotting and glycolysis for 48 hours, if the blood was stored

¹ Work done under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Rochester, and under contract with the Air Materiel Command, Wright Field.

² Each cc. containing 0.1 gram diallylbarbituric acid, 0.4 gram urethane, 0.4 gram monoethylurea, and q.s. distilled water.

in ice water. The withdrawal and storage of the blood was done without exposing it to air. The CO_2 and O_2 contents of 0.5 cc. arterial samples were measured by the Van Slyke and Neill (6) manometric technique of blood gas analysis. Arterial pH was determined at room temperature by the Beckman pH meter in a capillary glass electrode, without exposure to air. The hematocrit ratios were determined on duplicate samples of arterial blood, centrifuged exactly 20 minutes at 4000 r.p.m. The arterial blood of *dogs 4 to 10* was analyzed for lactic acid, according to the Barker and Summerson (7) technique, and the color concentration read by the Klett-Summerson colorimeter, using a 460 mu filter.

One-half hour before the beginning of asphyxia, the tibialis anterior and peroneus longus muscles in each hind leg of *dogs 8, 9, and 10* were dissected free, leaving blood and nerve supply intact. The skin flaps were closed to permit the reestablishment of normal gas content of the muscles. Two samples, each approximately 1 gram in size, were cut from each isolated muscle and the two corresponding samples from the other leg at death. The transfer of tissues to stoppered containers of CO_2 -free FeF_3 was made as quickly as possible (15 to 20 seconds from the time the skin flap was opened). Muscle CO_2 content was determined by the method of Danielson and Hastings (8), a modification of the Van Slyke manometric technique for blood gas analysis.

Electrocardiographic records (lead I only) were taken for 20 seconds out of every one or two minutes, i.e., at exactly the same time as blood and alveolar air were sampled by assistants in the experiment.

Muscles samples were analyzed within 18 hours after the experiment, and blood samples within 24 hours. The proteins and sugars were precipitated for the lactic acid determination within 24 hours, though completion of these analyses was often delayed up to 72 hours.

No attempt was made to resuscitate the animals, but blood samples were withdrawn as long as the arterial pressure would permit, and alveolar air samples were taken over the same length of time.

RESULTS

The mean values of each variable measured (with the exception of muscle CO_2), as well as their standard deviations, are presented in table 1. There was a wide variation from animal to animal in survival time; the mean figures for each variable therefore have progressively less reliability after the seven-minute interval. The same mean values are plotted in figure 1 as percentage change from the control values against time after the rebreathing began.

The mean curve of pulmonary ventilation reached its peak 4 minutes after rebreathing began. Respiration began to fail from $3\frac{1}{2}$ to 6 minutes, and completely ceased from $4\frac{1}{2}$ to $7\frac{1}{2}$ minutes after asphyxia began. Alveolar CO_2 continued to rise throughout the experiments, from a control mean of 6.48 percent to 14.10 percent at death. Every animal showed a progressive rise in alveolar CO_2 content.

Though the arterial pressure was not regularly recorded, in all cases it dropped so low that no more blood could be withdrawn. The average critical period be-

tween cessation of respiration and failure of blood pressure to the point where no more blood could be drawn was 2.7 minutes.

A mean rise in whole blood CO₂ content from 41.5 to 57.1, or a change of 15.6 vol. percent, occurred during the first 8 minutes, followed by a fall to 52.1, at the end of 10 minutes. The decreasing CO₂ content was shown by 7 of the 10 dogs reported here. Mean blood oxygen fell from a control level of 18.03 vol. percent to 0.33 vol. percent, 69.4 percent of the oxygen being lost during the

TABLE 1. MEAN EXPERIMENTAL DATA ON ASPHYXIATED DOGS

Each figure is an average of data from 8 to 10 dogs, unless otherwise stated. The standard deviations are given below each figure.

	NORMAL	2 MIN.	4 MIN.	6 MIN.	7 MIN.	8 MIN.	9 MIN.	10 MIN.
Respiration analyses								
% CO ₂ in alveolar air.....	6.48	8.44	10.07	11.83	13.80 ¹	13.67 ¹	14.17 ²	14.10 ²
σ.....	0.74	0.70	1.05	1.39	0.35	1.80	1.62	1.84
Alveolar pCO ₂ in mm. Hg.....	48.0	59.3	70.7	83.5	90.3 ²	95.1 ¹	97.8 ¹	98.5 ²
σ.....	4.51	4.35	7.22	8.95	13.14	12.86	12.24	24.36
Pulmonary ventilation in cc/min.	1153	3618	5236	3596 ²	2892 ²			
σ.....	280	198	2432	2462	634			
Arterial blood analyses: whole blood as drawn								
Vol % CO ₂	41.51	46.35	50.78	53.59	51.80 ²	57.07 ²	51.0 ²	52.1 ²
σ.....	5.55	4.97	5.43	6.03	2.40	2.99	8.63	4.36
Vol % O ₂	18.03	13.04	5.54	1.92	0.37 ²	0.99 ²	0.44 ²	0.33 ²
σ.....	2.70	1.73	3.35	1.82	0.31	0.44	0.14	0.18
pH 37°.....	7.26	7.22	7.19	7.13	7.09 ²	7.07 ²	7.07 ²	7.00 ²
σ.....	0.04	0.06	0.05	0.05	0.06	0.09	0.07	0.05
Hematocrit.....	39.4	37.7	41.2	43.9	49.2 ²	48.3 ²	47.6 ²	50.5 ³
σ.....	6.6	8.1	4.6	7.2	2.2	2.2	6.1	3.8
Lactic acid content in mEq/L..	1.22	1.15	1.49	2.08 ¹	2.92 ²	3.10 ²		3.40 ²
σ.....	0.58	0.45	0.47	0.85	0.80	1.28		1.85
Heart rate/min...	140	126	117	88	31 ²	94 ²	34 ²	
σ.....	30.3	35.5	30.9	41.1	17.0	45.3	10.3	

¹ 6 or 7 animals averaged. ² Less than 6 animals averaged. ³ 2 animals averaged.

first four minutes of asphyxiation, and 89.4 percent within the first six minutes. A temperature correction of -0.01 pH unit per 1°C. rise was applied to the pH values measured at room temperature (9). The results indicate a steady fall in arterial pH from a control mean of 7.26 to a terminal value of 7.00.

Control hematocrit values spread from 30.8 to 52.1 percent. As asphyxia progressed, however, the percentage in each dog rose, resulting in a mean increase of 28.2 percent during 10 minutes of asphyxia.

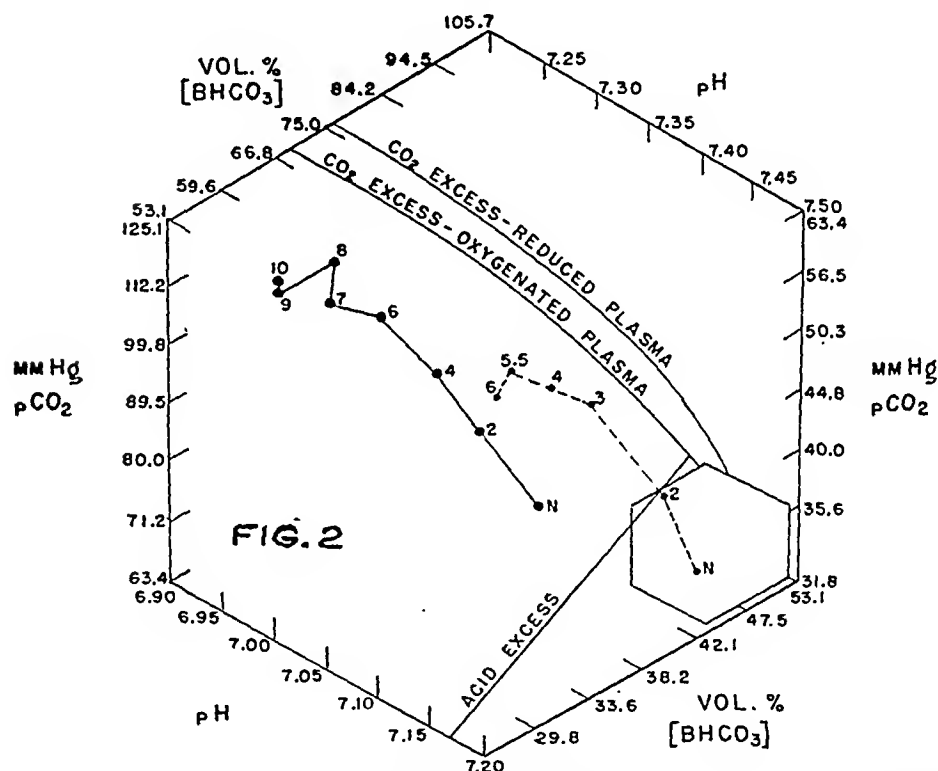
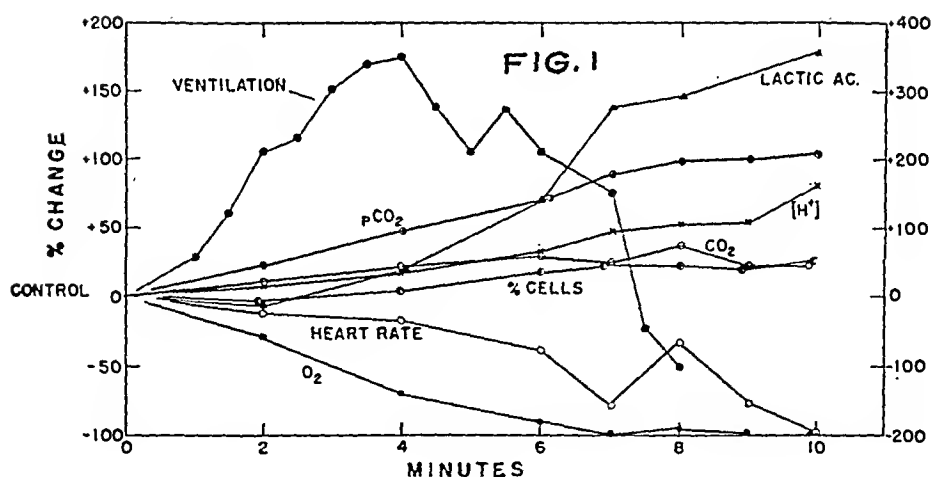


FIG. 1. SUMMARY OF VARIABLES OBSERVED DURING ASPHYXIA. The mean values of each variable are expressed as per cent change from the control. On the right is the scale for pulmonary ventilation. All other variables are plotted using the left hand scale.

Ventilation = cc. pulmonary ventilation/minute; $p\text{CO}_2$ = alveolar tension in mm.Hg; Lactic ac. = mg. lactic acid/100 cc. whole blood; O_2 = cc. O_2 /100 cc. whole blood; CO_2 = cc. CO_2 /100 cc. whole blood; $[\text{H}^+]$ = hydrogen ion concentration of plasma at 37°C .; % cells = hematocrit (4000 r.p.m. for 20 minutes); Heart rate = recorded by electrocardiogram.

FIG. 2. ACID-BASE BALANCE OF THE BLOOD DURING ASPHYXIA. The small interior hexagon represents the range of normal variation of dogs, as determined by Grodins, Lein, and Adler, this Journal 147: 433, 1946 (see text). The solid line represents the mean acid-base displacement of our dogs during asphyxia. (The calculations of the (BHCO_3) value of the last (10 min.) point by the Van Slyke and Sendroy nomogram make use of only two hematocrit values, separated by 8.9 per cent. Calculations using individual hematocrit values of 46.0 and 54.9 per cent give values of 52.6 and 54.4 (BHCO_3) respectively). The dashed line indicates the mean acid-base path of Grodins' asphyxiated dogs, reproduced from available data and graphs.

Lactic acid content of the arterial blood rose from a mean control level of 11 mgm. percent to 30.6 mgm. percent after 10 minutes of asphyxiation. The rise in lactic acid content did not become marked, however, until after about 6 minutes of asphyxiation, following which it rose very rapidly. This rise accompanied the fall of arterial oxygen below five volumes percent. This same relationship was pointed out by Scholander (10, 11) and Grinnell, Irving, and Scholander (12) on arrested breathing experiments with several species of animals.

The control heart rate, as recorded by the string galvanometer, was exceedingly variable from animal to animal, though the heart rate of each animal gradually decreased as asphyxia progressed. Cardiac change occurred, after respiration had ceased, usually in the form of auricular fibrillation and complete heart block.

TABLE 2. DOG MUSCLE CO₂ CONTENT—CONTROL AND DEATH FROM ASPHYXIA

EXPERIMENT NUMBER	MUSCLE ANALYZED	TOTAL CO ₂ IN VOL. PERCENT		COMBINED CO ₂ IN VOL. PERCENT		
		Normal	Death	Normal	Death	Av. Δ CO ₂
8	Tibialis anterior	36.5	31.6	33.9	26.4	
		29.0	25.0	26.4	19.8	-7.0
	Peroneus longus	32.8	37.0	30.2	31.8	
		35.6	29.9	33.0	24.7	-3.3
9	Tibialis anterior	36.7	37.1	33.6	30.8	
		34.2	37.2	31.1	30.9	-1.5
	Peroneus longus	33.4	38.6	30.3	32.3	
		38.0	29.1	34.9	22.8	-5.0
10	Tibialis anterior	—	33.1	—	27.4	
		33.8	33.5	31.0	27.8	-3.2
	Peroneus longus	36.2	35.9	33.4	30.2	
		37.9	38.6	35.1	32.9	-2.7
Mean.....		34.9	33.9	32.1	28.2	
Average Δ CO ₂						-3.8

A supraventricular tachycardia that cannot further be defined is the cause of the increase in mean heart rate at the eight-minute interval.

Complete muscle data are found in table 2. The mean difference between samples of the same muscle is 3.04 vol. percent, and the σ of the difference is 2.12. Total CO₂ was analyzed directly by the Danielson and Hastings technique. The combined CO₂ content was obtained by subtracting from the total CO₂ content the amount of CO₂ dissolved at the pCO₂ to which the muscle was subjected. This assumes a CO₂ absorption coefficient in muscle of 0.41, after Irving, Foster, and Ferguson (13). Muscle pCO₂ was estimated as arterial (alveolar) pCO₂ plus 6 mm. Hg (A-V difference), on the doubtful assumptions that the A-V pCO₂ difference is a constant in spite of different circulation rates, and that muscle pCO₂ is the same as venous pCO₂.

DISCUSSION

Our experimental data concerning the acid-base balance of the blood, as expressed by the Henderson-Hasselbalch equation, $\text{pH} = \text{pK}_1 + \frac{\log (\text{BHCO}_3)}{\log (\text{H}_2\text{CO}_3)}$, have been plotted as a solid line on the logarithmic triaxial graph (fig. 2) introduced by Shock and Hastings (14). The inner hexagon represents the normal acid-base range of dog blood determined by Grodins *et al.* The pathways of CO_2 excess represent averages of *in vitro* CO_2 absorption curves of oxygenated and reduced true plasma (Shock and Hastings, 15; taken from the data of Henderson, 16). These pathways, as well as the fixed acid excess path, are in accordance with *in vivo* experimental observations of Shock and Hastings (15).

Our data are plotted from alveolar pCO_2 , measured directly, and plasma (BHCO_3), calculated from whole blood (BHCO_3) by the Van Slyke and Sendroy (17) nomogram. This nomogram makes use of mean hematocrit values for each time interval. The mean figure for the last (10 min.) point is an average of only two hematocrit values, separated by 8.9 percent. Nomogram calculations making use of the individual hematocrits, 46.0 and 54.9 percent, give values of 52.6 and 54.4 vol. percent (BHCO_3), respectively.

It will be noted that the pH values so determined agree within 0.05 pH unit with experimental pH values corrected to 37°C ., (with the exception of the mean figure for the nine-minute interval). A depression of the respiratory center, due to anesthesia alone, must account for the relatively acid pH and high pCO_2 of the 'normal' sample.

The acid-base displacement curve of our dogs agrees very closely with that of Grodins *et al.*, who asphyxiated dogs by completely obstructing the trachea, and whose results are shown approximately as a dashed line on figure 2.

Our data, with those of Grodins *et al.*, can best be described as representing an initial respiratory acidosis, associated with decreasing pH, increasing (BHCO_3), and increasing pCO_2 . Superimposed on the respiratory acidosis toward the end of asphyxia is a slight uncompensated metabolic acidosis, characterized by a decreasing pH, decreasing (BHCO_3), and increasing pCO_2 . The source of metabolic acidosis in these experiments appears to be an accumulation of lactic acid and other fixed acids from the anaerobic glycolysis taking place in the absence of oxygen, and displacing CO_2 from the blood. The accumulation of lactic acid in the blood is shown by the direct measurements of lactic acid content indicated in table 1.

Approximately 2 percent of the 28.2 percent rise in hematocrit can be accounted for by the shifting of water from plasma to cells, accompanying the acid-base changes of the blood during the experiment (16). The remaining 26.2 percent is explained by two possible mechanisms, neither of which was actually observed in these experiments, but both of which are known to occur in asphyxia: 1) discharge of red blood cells from the spleen into the circulation in response to lowered oxygen tension and 2) leakage of fluid and whole plasma into tissues, and a consequent hemoconcentration associated with shock. Thompson and

Birnbaum (2) have found regular evidence of shock in dogs subjected to obstructive asphyxia. Grodins *et al.* report an increase in hemoglobin content of 24.6 percent during obstructive asphyxia in dogs.

Inspection of table 3 reveals that the CO₂ content of the muscles is no higher at the end of asphyxia than at the beginning, as would be the case if these muscles stored the CO₂ displaced from the blood; rather, the mean death sample CO₂ content is 3.8 vol. percent lower than the control, despite increasing CO₂ tensions in the muscles. Each figure for muscle CO₂ content in table 3 is an average of four CO₂ analyses: two of tibialis anterior and two of peroneus longus. By comparison of our muscle CO₂ data with the CO₂ dissociation curve and muscle buffering power data found for dog muscle by Irving, Foster, and Ferguson, we have estimated the amount of fixed acid necessary to account for the decrease in muscle CO₂ content, despite increased muscle pCO₂, as asphyxia progressed. For this purpose it has been assumed, as previously, that muscle pCO₂ equals arterial (alveolar) pCO₂ plus 6 mm. Hg. Muscle pH is calculated from the Henderson-

TABLE 3. SUMMARY OF DOG MUSCLE EXPERIMENTS

EXPERIMENT NUMBER	COMBINED CO ₂ IN MUSCLE	ARTERIAL pCO ₂ PLUS 6 mm. Hg	MUSCLE pH	ESTIMATED LACTIC ACID CONTENT OF MUSCLE AT DEATH	LACTIC ACID CONTENT OF ARTERIAL BLOOD AT DEATH
	<i>Vol %</i>	<i>mm. Hg</i>		<i>mEq/L.</i>	<i>mEq/L.</i>
8 Control.....	30.9	48.5	7.17		
Death.....	25.7	95.9	6.79	9.65	3.40
9 Control.....	32.5	56.6	7.12		
Death.....	29.2	116.0	6.80	8.68	5.24
10 Control.....	33.2	51.0	7.18		
Death.....	29.6	105.4	6.85	9.05	2.71

Hasselbalch equation, the pK₁ value being 6.1. Recent evidence of Conway and Fearon (18) for carbamino-bound CO₂ in muscle would make these pH values considerably lower, though the relative changes would be the same.

The estimated muscle lactic acid content at death (table 3, column 5) varies from 8.68 to 9.65 mEq./liter. Simultaneous lactic acid contents of the blood (table 3, column 6) range from 2.71 to 5.24 mEq./liter, or approximately one-third to three-fifths the muscle lactic acid values. This is evidence that arterial lactic acid, at the time the circulation ceases, has not come into equilibrium with muscle lactic acid, which is formed by anaerobic glycolysis, and diffuses outward into the circulation. These figures furnish a measure of the degree of metabolic acidosis existing in the tissues at the time of death from asphyxia.

At the time of respiratory failure from asphyxia in man, if the CO₂ concentration in the alveolar air is as high as it averaged in the dogs reported here—11 to 13 percent—CO₂ narcosis would be an important factor in respiratory failure. Blood CO₂ content would be so high that the respiratory center would presumably be depressed; vasodilatation and a fall in blood pressure would ensue (19).

It is therefore questionable, whether, under such circumstances, administration of carbogen would have the beneficial effect desired. Rather, it would seem that the additional CO_2 would serve to narcotize the respiratory center to an even greater degree. Until the CO_2 tension of the blood was reduced below the narcotic level, no beneficial effect could be derived from the added CO_2 . Before the 'safe' level of CO_2 was attained in the blood, the critical period between cessation of respiration and heart change (about 30 seconds) would possibly have passed; efforts at resuscitation are of no avail a few seconds after the heart has stopped.

SUMMARY

1. Ten healthy anesthetized dogs were asphyxiated by rebreathing limited amounts of room air. A continuous pulmonary ventilation record was made, and electrocardiographic records, arterial blood, and alveolar air samples were taken simultaneously at one- or two-minute intervals during asphyxiation. Analysis of arterial CO_2 , oxygen, and lactic acid content, pH, and hematocrit percentages were made. Alveolar samples were analyzed for CO_2 content. Control and death samples of muscle were analyzed for CO_2 .

2. The acid-base balance of the blood responded to asphyxia with a respiratory acidosis, and a late metabolic acidosis superimposed. Direct measurements indicated that incoming lactic acid is the cause of CO_2 displacement from the blood during metabolic acidosis.

3. Muscle CO_2 content of dogs is slightly lower at the time of death from asphyxia than it is normally. This decrease in CO_2 content is a measure of the diffusion gradient of lactic acid from tissues to blood and of the degree of metabolic acidosis in the tissues during asphyxia.

4. The initial use of carbogen in resuscitating men subjected to any form of obstructive asphyxia is questioned.

The writer is deeply indebted to Professor Wallace O. Fenn and Dr. Arthur B. Otis for their counsel and constant encouragement throughout this research.

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OBSERVATIONS OF ARTERIAL OXYGEN CONTENT IN CHILDREN DURING THE INHALATION OF AIR AND 100 PER CENT OXYGEN¹

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Received for publication January 29, 1948

Analyses of arterial blood from 14 normal children have been carried out to determine the percentage oxygen saturation while breathing air and the amount of dissolved oxygen in the blood during the inhalation of 100 per cent oxygen. This investigation was undertaken to provide normal data necessary for the interpretation of studies on children with congenital heart disease. In addition the results of the study appear to indicate that the disparity between observed and theoretical values for arterial oxygen saturation has not yet been adequately explained.

METHODS

Selection of subjects. The children were ambulatory hospital patients without any evidence of cardiac or pulmonary disease. There were 8 boys and 6 girls, whose ages ranged between 7 and 14 years. Studies were carried out on five subjects prior to elective plastic or ocular surgery; on one, three days following an eye muscle operation, and on four others between the seventh and ninth days following appendectomies. One patient had been admitted with diabetes mellitus, one with congenital syphilis, one with possible chorea, and one with possible encephalitis; all were ready for discharge from the hospital when blood was taken for this study.

Technique of arterial puncture. The femoral artery was employed in all cases. The arterial site was determined by palpation, which was facilitated by holding the elected thigh in a position of slight external rotation while the subject was supine. This area, approximately one inch distal to the inguinal ligament, was infiltrated with 2 to 3 cc. of 2 per cent procaine without epinephrine. The puncture was made by using an ordinary lumbar puncture needle provided with a blunted stylet: a #19 gauge needle, $2\frac{1}{2}$ inches in length, was suitable for the older children, whereas a #20 gauge needle, about $1\frac{1}{4}$ inches in length, was preferable for the younger ones. The needle was attached to a 2 cc., all-glass syringe which had been lubricated previously with paraffin oil and moistened with sufficient 10 per cent potassium oxalate solution to fill the dead space, in the manner described in another communication (1). It was inserted at a 45-degree angle through the anesthetized skin in the line of pulsation; as soon as

¹ This investigation was aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

² The data reported in this paper are from a thesis to be presented by this author in partial fulfillment of the requirements for the degree of Doctor of Medicine.

the needle entered the artery the plunger of the syringe began to move backwards. Approximately 2 cc. of blood were withdrawn and then the syringe was disconnected from the needle and replaced by the stylet. Immediately following the removal of each blood sample the syringe was placed in an ice and water bath until the time of analysis (1). After the final blood sample had been collected and the needle removed from the artery, a dry sponge was held firmly against the site of puncture for several minutes to prevent the formation of a hematoma.

Experimental conditions. All of the selected patients were cooperative and were in a resting state before and during the collection of the blood samples.

1. Subject breathing air. About 10 minutes after the child assumed the recumbent position the arterial puncture was made and the first 2 cc. blood sample was withdrawn.

2. Subject breathing oxygen. While the obturated needle was in the artery, the child breathed 100 per cent oxygen from a snugly fitting, soft rubber mask³ which was held over the mouth and nose. The gas was delivered from a cylinder at a rate sufficiently rapid to keep the reservoir bag distended throughout the respiratory cycle, thus precluding any leak of air into the system. An inspiratory valve interposed between the reservoir bag and mask and an expiratory valve situated on the dome of the mask operated with negligible resistance and virtually eliminated rebreathing. Oxygen was supplied for a period of three minutes, which was considered sufficient time for the elimination of most of the nitrogen from the lungs (2, 3, 4). It has been shown that after 35 to 40 breaths of normal respiratory volume the alveolar nitrogen is reduced to 2 per cent (3), and that the nitrogen content of the arterial blood is at a constant low level within 2½ minutes (4). The subject continued to breathe oxygen until the second blood sample had been collected.

Analytical procedure. A modified Roughton-Scholander technique of gasometric analysis (1, 5), using 0.04 cc. of blood, was employed in the determination of the oxygen content and capacity of each sample. Duplicate analyses were run simultaneously by two technicians; observed values which checked within 0.2 vol. per cent before reduction to standard conditions were considered satisfactory. The determination of oxygen content was begun as soon as possible on the second sample; there was usually an interval of 15 to 20 minutes between drawing and analyzing the blood. The first sample, collected while the subject was breathing air, was then analyzed for oxygen content. About two hours after the blood had been drawn, a 0.5 cc. aliquot was transferred to a 15 cc. bottle and equilibrated with air for 30 minutes on a mechanical rotator (1), and then the oxygen capacity was immediately determined.⁴

Calculations. Values for oxygen content and capacity were expressed in

³ Adult Oro-Nasal Meter Mask (Type #720), used without the meter attachment. It is distributed by the Oxygen Equipment Manufacturing Company, 405 East 62nd Street, New York, New York.

⁴ In cases 1 and 2 of tables 1 and 2 the blood samples were saturated with oxygen before the equilibration with air, which was accomplished by hand rotation. It is possible that equilibrium between blood and air was not reached in these two cases.

volumes per 100 cc. of blood under standard conditions of temperature and barometric pressure. From the observed total oxygen capacity the amount of oxygen carried in physical solution (d_{air}) was subtracted (1). The values for total oxygen content and corrected oxygen capacity were increased by 2 per cent to correct for the dilution of the blood sample by the oxalate solution used to fill the dead space of the syringe when the blood was drawn (1). Percentage oxygen saturation was calculated only on the blood drawn while the subject was breathing air, according to the equation,

$$O_2 \text{ saturation (\%)} = 100 \times \frac{\text{Total } O_2 \text{ content} - \text{dissolved } O_2}{\text{Corrected } O_2 \text{ capacity}}$$

in which the value for dissolved oxygen in the numerator was taken from a table published elsewhere (1).

Dissolved oxygen (d) is defined as the amount of oxygen in cubic centimeters per 100 ml. of whole blood carried in physical solution in the circulating arterial blood, d_{air} and d_{O_2} representing dissolved oxygen during the inhalation of air and 100 per cent oxygen, respectively. The quantity of dissolved oxygen observed during the inhalation of 100 per cent oxygen, $d_{O_2 \text{ obs.}}$ is equal, by definition, to the total oxygen content minus the corrected oxygen capacity.

EXAMPLE: Case 14 (see table 2)

Total O_2 content = 19.38 vol. per cent

Corrected O_2 capacity = 18.69 vol. per cent

$d_{O_2 \text{ obs.}}$ = 0.69 vol. per cent

The amount of dissolved oxygen that would theoretically be present in a sample of blood equilibrated with oxygen at a tension equal to that in the alveolar air of a person breathing 100 per cent oxygen, is designated $d_{O_2 \text{ calc.}}$ and is calculated as follows:

1. The Bunsen solubility coefficient of oxygen at 38° , α_{38° , is determined for a blood sample of any corrected oxygen capacity according to the following empirical equation of Sendroy, Dillon, and Van Slyke (6):

$$\alpha_{38^\circ} = 0.0209 + 0.000108 (\text{corrected oxygen capacity in vol. \%})$$

2. The value of α_{38° thus determined is substituted in the formula,

$$d_{O_2 \text{ calc.}} = \frac{100 (B - 87) \alpha_{38^\circ}}{760}$$

in which $d_{O_2 \text{ calc.}}$ is dissolved oxygen in volumes per cent, and the expression, $B - 87$, represents the observed barometric pressure corrected for the tensions of CO_2 (40 mm. Hg) and water vapor (47 mm. Hg) assumed to be present in alveolar air. The small amount of residual nitrogen in the alveoli has been ignored.

Values for $d_{O_2 \text{ obs.}}$ and $d_{O_2 \text{ calc.}}$ were determined for each patient. The difference between them in volumes per cent, $d_{O_2 \text{ calc.}} - d_{O_2 \text{ obs.}}$ appears as Δ in table 2.

The data on blood samples withdrawn during the inhalation of air appear in table 1, and table 2 summarizes the observations made on samples removed while the subjects were breathing oxygen. Values for total oxygen content do not appear in table 2; they are equal to the sums of corresponding values for $d_{O_2 \text{ obs.}}$ and corrected oxygen capacity.

RESULTS

The average arterial oxygen saturation of the 14 normal children while breathing air at rest was 93.1 per cent, with a range of 91.5 per cent to 95.3 per cent (see

TABLE 1. ARTERIAL OXYGEN STUDIES IN NORMAL CHILDREN WHILE BREATHING AIR

CASE	TOTAL O ₂ CONTENT	CORRECTED O ₂ CAPACITY	O ₂ SATURATION	CASE	TOTAL O ₂ CONTENT	CORRECTED O ₂ CAPACITY	O ₂ SATURATION
	vol. %	vol. %	%		vol. %	vol. %	%
1	19.8 ₄	20.9 ₁	93.8	9	18.0 ₂	19.2 ₄	92.6
2	15.6 ₆	16.8 ₆	91.7	10	16.4 ₇	17.2 ₉	93.9
3	15.9 ₆	17.1 ₈	91.7	11	15.6 ₈	16.6 ₂	92.8
4	17.4 ₇	18.3 ₈	93.8	12	15.8 ₀	16.3 ₂	95.3
5	14.5 ₄	15.4 ₄	92.7	13	14.6 ₀	15.7 ₄	91.5
6	15.0 ₆	15.8 ₈	93.6	14	17.5 ₇	18.4 ₉	93.8
7	16.0 ₇	16.8 ₈	93.3				
8	14.4 ₃	15.4 ₃	92.5	AVERAGE	16.23	17.19	93.1

TABLE 2. DISSOLVED OXYGEN IN THE BLOOD OF NORMAL CHILDREN DURING THE INHALATION OF 100 PER CENT OXYGEN

CASE	CORRECTED O ₂ CAPACITY	dO ₂		
		dO ₂ obs.	dO ₂ calc.	Δ
	vol. %	vol. %	vol. %	vol. %
1	20.0 ₁	1.0 ₃	2.0 ₆	1.0 ₂
2	16.5 ₁	0.9 ₅	1.9 ₇	1.0 ₂
3	16.9 ₁	1.0 ₈	2.0 ₁	0.9 ₃
4	16.3 ₇	1.0 ₉	2.0 ₀	0.9 ₁
5 ¹	(12.7 ₆)	(0.9 ₃)	(1.9 ₇)	(0.9 ₈)
6	16.0 ₈	1.1 ₁	1.9 ₉	0.8 ₈
7	16.9 ₁	1.0 ₀	2.0 ₁	1.0 ₁
8	15.1 ₀	1.6 ₆	1.9 ₈	0.3 ₂
9	19.3 ₆	0.8 ₉	2.0 ₂	1.1 ₃
10	17.0 ₂	1.4 ₆	2.0 ₂	0.5 ₆
11	16.7 ₀	1.0 ₆	2.0 ₂	1.0 ₂
12	16.2 ₁	1.3 ₄	2.0 ₀	0.6 ₆
13	15.6 ₄	0.9 ₂	1.9 ₈	1.0 ₆
14	18.6 ₉	0.6 ₉	2.0 ₁	1.3 ₂
AVERAGE.....	17.04	1.09	2.00	0.91
STANDARD DEVIATION.....		±0.26		±0.26

¹ The results of analyses in *Case 5* were not included in the averages because of possible error due to blood blotting.

table 1). During the inhalation of oxygen the total oxygen content usually increased by about 2 vol. per cent, and in all cases the apparent oxygen saturation reached 100 per cent. In *case 4* there was no absolute increase in oxygen content during the inhalation of oxygen; there was, however, a decrease of ap-

proximately 2 vol. per cent in the oxygen capacity of the second blood sample, so that the oxygen saturation was complete.

The average value for the observed amount of dissolved oxygen in the blood during the inhalation of pure oxygen was 1.09 vol. per cent (see table 2). The result of the determination of $d_{O_2 \text{ obs.}}$ in case 8 seems disproportionately high, though the analyses appeared satisfactory; if this case were omitted the average $d_{O_2 \text{ obs.}}$ would equal 1.05 vol. per cent.

The theoretical value for the quantity of dissolved oxygen was in each case higher than actually observed. This discrepancy, indicated by Δ , averaged 0.91 vol. per cent.

DISCUSSION

The average figure for oxygen saturation, 93.1 per cent, is 0.8 to 2.9 per cent lower than other reported averages (7-12). One of the earlier investigations (7), in which a colorimetric method was employed, was carried out on 28 normal children; an average value of 94.7 per cent was reported with a range of 91 to 98 per cent. In the other studies, carried out on adults, standard gasometric procedures were used, but it is not always clear from the reports that corrections were made for dissolved oxygen. In the present study an average figure of 0.22 vol. per cent for dissolved oxygen was subtracted from the total oxygen content; had this correction not been made our average saturation would have been calculated as 94.4 per cent. All of the children were ambulatory, and many were given deep breathing exercises before the arterial sampling procedure was begun. Any reduction in percentage saturation brought about by deficient pulmonary ventilation and atelectasis induced by bed rest (3, 9) was thus avoided.

Our average arterial saturation of 93.1 per cent is 5.5 per cent lower than the average value of 98.6 per cent determined spectrophotometrically by Drabkin and Schmidt (13) on freshly drawn, oxalated, hemolyzed, human arterial blood taken while the subjects were breathing air. Our average difference of 0.91 vol. per cent between $d_{O_2 \text{ obs.}}$ and $d_{O_2 \text{ calc.}}$ indicates that during the inhalation of oxygen, the observed oxygen content was likewise lower than its predicted value, by an average 5.3 per cent. The discrepancy between empirical and theoretical values for arterial oxygen saturation has been attributed by Roughton, Darling, and Root (11) to factors inherent in the gasometric determination of oxygen capacity. The two factors of importance are differential drainage of cells and plasma from the saturating vessel and spontaneous reversion of a fraction of the hemoglobin from a form which is inactive *in vivo* but becomes capable of combining with oxygen after being withdrawn from the body. In the present study drainage factors were held to a minimum in the determination of oxygen capacity, leaving the reversion of inactive hemoglobin as a plausible explanation for the discrepancies in our figures. However, actual measurements in other laboratories of the carbon monoxide-combining power of blood immediately after its removal from the body and again an hour or two later indicate an average spontaneous increase in carbon monoxide capacity of only 0.4 (14) to 1.1 (11) per cent.

The answer to the problem of discrepancies between observed and theoretical contents of oxygen in the circulating arterial blood may be resolved by a study in which determinations of arterial oxygen saturation by both gasometric and spectrophotometric methods are carried out on the same specimens of blood. The studies of arterial oxygen tension carried out by Comroe and Dripps on patients breathing air (15) and by Berggren on individuals breathing 100 per cent oxygen (3) leave little doubt that arterial blood is normally in equilibrium with alveolar air. The hypothesis of Roughton, Darling, and Root (11), that deviation of gasometric results from the theoretical must be attributed to changes in stored blood, is thus supported by good indirect evidence. However, their explanations for these changes reconcile the entire discrepancy neither in the present study nor in those investigations in which higher average values for arterial oxygen saturation are reported. Apparently the manipulations attendant to saturating blood with oxygen or carbon monoxide bring about an increase in the gas-combining capacity of the blood.

It is apparent from table 2 that over the small range of oxygen capacities observed in this study—viz., 15–20 vol. per cent—there is no demonstrable correlation between oxygen capacity and $d_{O_2 \text{ obs.}}$. Theoretical variations in d_{O_2} with oxygen capacity would, however, be too small to be detected by the analytical techniques we have employed, as is evident from the values for $d_{O_2 \text{ calc.}}$ in table 2.

The results of these experiments find practical application in the diagnosis of congenital heart disease. Knowledge of the average amount of oxygen carried in physical solution in the pulmonary venous blood during the inhalation of oxygen has made it possible to estimate quantitatively the extent of impairment of pulmonary diffusion of oxygen as well as the shunting of venous blood into the systemic circulation, in patients with arterial anoxemia (16).

It must of course be borne in mind that the values here reported are defined in terms of the analytical techniques employed.

SUMMARY

A technique of femoral artery puncture has been described. Arterial blood samples were obtained from 14 normal children and the results of oxygen analyses by a modified Roughton-Scholander technique have been reported.

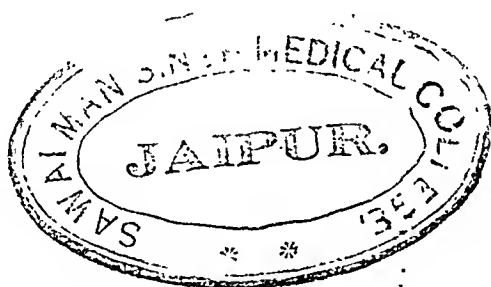
The average arterial oxygen saturation under conditions of rest and the breathing of room air was found to be 93.1 per cent, with a range of 91.5 to 95.3 per cent.

The average amount of oxygen in physical solution in the arterial blood during the inhalation of 100 per cent oxygen was found to be 1.09 vol. per cent, or 55 per cent of the amount theoretically expected. The difference between the theoretical and observed values is of the same order of magnitude as the discrepancy between the theoretical and observed values for arterial oxygen saturation during the inhalation of air. The possible mechanisms that produce these discrepancies have been discussed.

We wish to express our deep appreciation and sincere thanks to Miss Dorothy Nixon for her technical assistance and cooperation in this project.

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THE AMERICAN JOURNAL OF PHYSIOLOGY

Published by
THE AMERICAN PHYSIOLOGICAL SOCIETY

VOLUME 153

April 1, 1948

NUMBER 1

PROOF OF A HORMONAL MECHANISM FOR GASTRIC SECRETION—THE HUMORAL TRANSMISSION OF THE DISTENTION STIMULUS¹

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PROOF for the existence of a hormonal mechanism for gastric secretion has, until now, been lacking. Strong but nevertheless inconclusive evidence for the existence of such a hormone has long been available, but evidence that can be considered crucial has not been adduced.

The status of the problem up to the time of the present studies has been clearly and extensively reviewed (1-4). In broad outline it can be summarized as follows.

A humoral mechanism for gastric secretion has been proven to exist (5) by the demonstration that the feeding of a meal produces a secretory response in a subcutaneously transplanted (and thus extrinsically denervated) gastric pouch. This has raised the question of whether the stimulation of the transplanted portion of the stomach by food in the main stomach is due to absorption of certain constituents of the food into the blood stream or to the formation of a hormone by the gastric mucosa under the influence of contact with stimulating chemicals in food.

The further analysis of the humoral mechanism has involved the study of the site of origin of the humoral agent and the nature of this agent. As regards the site of origin, it has been demonstrated that a humoral agent can arise from both the stomach and the intestine (4). In the present studies we are concerned only with the humoral agent arising in the stomach.

Two kinds of stimuli acting in the stomach are effective in evoking gastric secretion; namely, chemical stimuli and mechanical (distention) stimuli. That the chemical stimuli can be humorally transmitted has been well known. In the only previous study in which an attempt was made to determine whether the distention stimulus is humorally transmitted, the results indicated that it was

Received for publication April 7, 1948.

¹ A preliminary report of this work was read before the XVIIth International Physiological Congress, Oxford, England, July 1947.

not (4). Therefore all previous considerations of the nature of the humoral agent have dealt only with the chemical stimuli and have been primarily concerned with the question of whether the humoral agent is absorbed secretagogues or is a hormone the release of which from the gastric mucosa into the blood stream is caused by the secretagogue.

Inasmuch as no secretagogue is known which will stimulate gastric secretion when placed in the stomach but will not stimulate when injected intravenously, the possibility must be entertained that absorbed secretagogues are the humoral agent. However, it has been well established (5) that secretagogues are stronger secretory stimulants for a transplanted gastric pouch when perfused through the main stomach than when introduced intravenously. This evidence favors the view that not all of the stimulatory action of secretagogues on such a transplanted preparation is due to their absorption into the blood stream and suggests that the secretagogue may also cause the release of a hormone. The other possible explanation of these facts is that the gastric juice acts upon the secretagogue in some way to increase its stimulatory action when it is absorbed. The fact that Kim and Ivy (6) found no augmentation of stimulation by incubation of secretagogue (liver extract) with gastric juice speaks against this latter possibility.

Distention Stimulus for Gastric Secretion

The question of whether distention of the stomach stimulates a secretory response was considered controversial until Lim, Ivy and McCarthy (7) unequivocally demonstrated the existence of such a mechanism. The literature up to 1925 on this subject has been comprehensively reviewed by these authors and we shall only refer here to those studies which have a bearing upon the mechanism of the distention stimulus. The distention stimulus has been shown to occur in the stomach completely deprived of extrinsic nervous connections and even in some instances in the subcutaneously transplanted stomach (8, 9). It has also been shown that distention of the pyloric portion of the stomach will induce secretion of acid in the fundic glands of a pouch even after the extrinsic nerves to this pouch have been severed (7). The interpretation placed upon this observation has varied. Lim, Ivy and McCarthy favored the view that a vasomotor effect transmitted the distention stimulus between these two portions of the stomach unconnected by nerves, whereas Chang and Lim (10) considered it to indicate a hormonal mechanism. However since the classical work of Ivy and Farrell (8), it has generally been recognized that in order to prove that any particular mechanism of gastric secretion is humorally transmitted it is necessary to demonstrate that mechanism by the use of the subcutaneously autotransplanted pouch. Unless the transplantation technique is used the possibility of participation of extrinsic nerves cannot be eliminated with certainty regardless of the thoroughness with which the denervation is done.

One attempt to determine whether distention of the main stomach would evoke secretion in the transplanted fundic pouch has been made by Gregory and Ivy (4). These workers observed no secretory response under the conditions of their experiments. Inasmuch as it had been clearly shown by a number of workers (11, 10, 7) that the distention stimulus could be transmitted between two separated portions of the stomach, these negative results tended to indicate that this transmission depended upon nervous pathways which had not been completely severed in the earlier experiments but which were unquestionably interrupted in the transplantation operation.

In experiments of this type a positive result is crucial whereas a negative result might be due to technical difficulties. Because several possible sources of such technical imperfections were recognized in the studies of Gregory and Ivy, the present work was undertaken to reinvestigate the problem.

METHODS

Several different kinds of animal preparation were used in this study. These will be described individually.

GROUP 1. *Subcutaneously transplanted fundic pouch with vagally denervated pouch of the remainder of the stomach.* This is essentially the same preparation used by

Gregory and Ivy (4) with the exception that in the animals used in the present studies a much larger portion of the fundic portion of the stomach was transplanted.

In two animals (*no. 1* and *2*) the operative procedure was the same as that used by Gregory and Ivy. At the first operation the fundic pouch was placed under the skin and its vascular pedicle was permitted to remain intact. From one third to one half of the fundic portion of the stomach was used for making the pouch, so that these pouches were from two to three times the size of those used by Gregory and Ivy. The second operation, performed four to eight weeks after the first operation, consisted

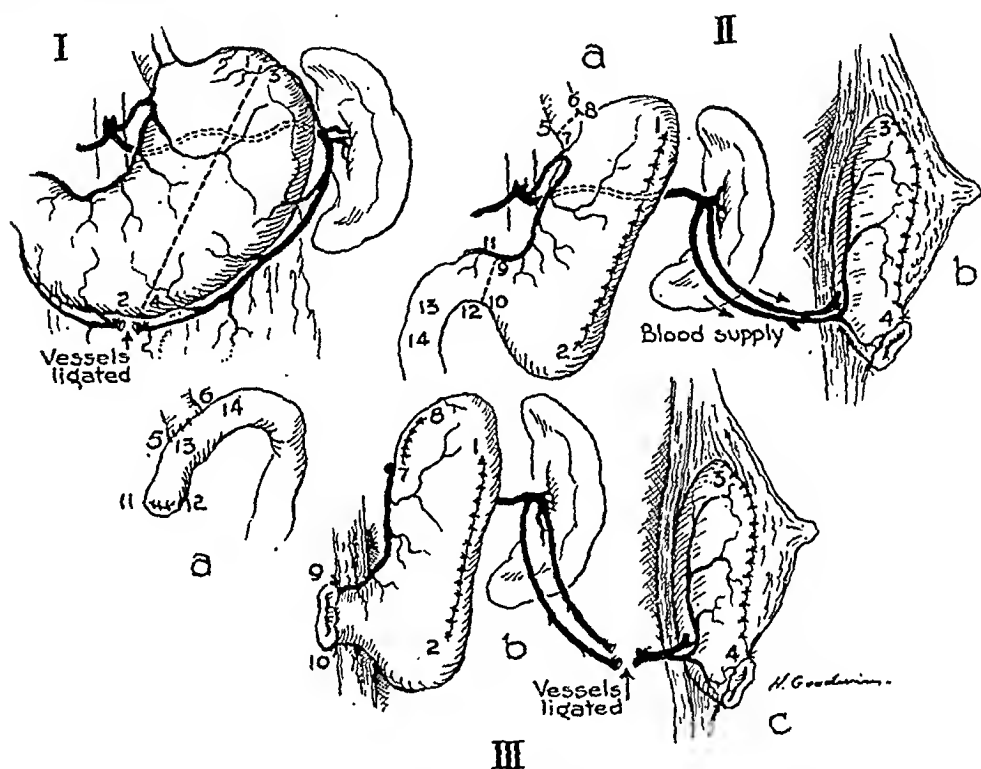


Fig. 1. SKETCH OF OPERATIVE PROCEDURE used in preparing a 'two-pouch dog' with a transplanted fundic pouch and a pouch of the remainder of the stomach. I. Normal stomach showing line of incision and point of ligation of greater curvature vessels. II. First-stage operation: *a*) remnant of stomach showing lines of incision to be used in the second-stage operation; *b*) subcutaneously transplanted fundic pouch. III. Second-stage operation: *a*) esophagoduodenostomy, *b*) pouch of remainder of stomach; *c*) transplanted fundic pouch with vascular pedicle ligated.

of ligation and transection of the pedicle to the subcutaneous pouch and formation of a pouch from the remainder of the stomach. The latter was accomplished by transection at the pyloro-duodenal junction and at the esophago-gastric junction followed by esophago-duodenal anastomosis to restore intestinal continuity. The fundic end of the stomach was closed and the pyloric end was brought out through a stab wound to serve as a stoma. Figure 1 is a sketch of the steps in the operative procedure.

In one animal (*no. 3*) the operative procedure was reversed in that a pouch of the entire stomach was made at the first operation. At the second operation the stomach

was transected at the level of the incisura angularis and the entire fundic portion was placed in a subcutaneous pocket, allowing the vascular pedicles to remain. Six weeks later a third celiotomy was performed at which the vascular pedicles were ligated and transected. In this animal almost the entire acid-secreting portion of the stomach was incorporated in the transplanted pouch.

In still another animal (*no. 4*) after performing the first stage as in animals *no. 1* and *2*, at the second operation the stomach was transected at the level of the incisura

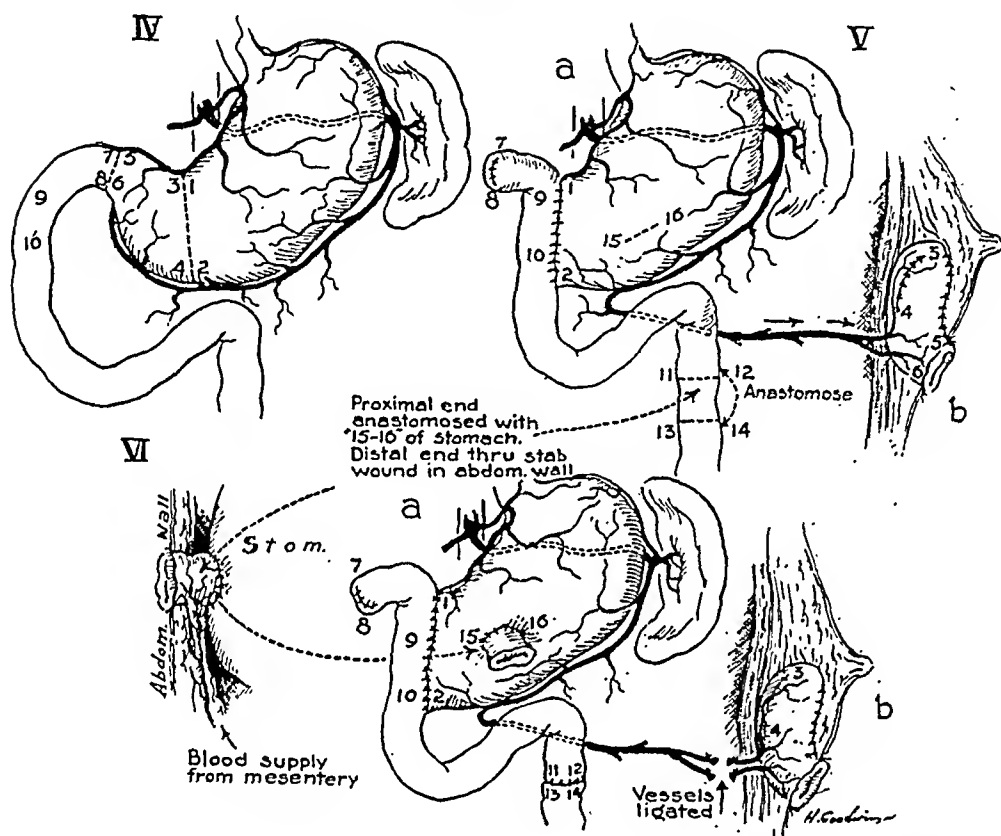


Fig. 2. SKETCH OF OPERATIVE PROCEDURE used in preparing a dog with a transplanted pyloric pouch and a gastric fistula. IV. Normal stomach showing lines of incision for first-stage operation. V. First-stage operation: a) main stomach with gastroduodenostomy; b) subcutaneously transplanted pyloric pouch. VI. Second-stage operation: a) main stomach with Mann-Bollman type fistula; b) transplanted pyloric pouch with vascular pedicle ligated and transected.

angularis and at the pyloric sphincter. The lower portion of the stomach was fashioned into a pouch and the upper end was anastomosed to the duodenum. In this animal the intra-abdominal pouch was formed almost entirely by the pyloric portion of the stomach. Some acid-secreting glands were, however, included in it.

GROUP II. *Subcutaneously transplanted pyloric pouch and gastric fistula.* Two dogs (*no. 5* and *6*) of this type were prepared. The first stage operation consisted of transection of the stomach at the level of the incisura angularis and again at the pyloric sphincter. The vessels along the greater curvature were not divided and they served as the sole vascular supply to this pyloric pouch which was placed in a subcutaneous pocket in the same manner as the fundic pouches described above. At a

second operation, 3 to 6 weeks later, this vascular pedicle was transected between ligatures and a fistula of the Mann-Bollman type (12) was made into the lower ventral surface of the main stomach utilizing a small segment of jejunum as the fistula path. This operation is sketched in figure 2.

Basal secretion. All animals were fasted for at least 12 hours before each experiment. In each experiment the basal secretion was collected, measured and titrated for from one to six hours before distention was performed. This permitted a comparison to be made in order to determine whether the rises in acid output during spontaneous fluctuations in the basal secretion were ever as great as those occurring in response to distention.

Method of distending. Condom balloons tied to the ends of pieces of rubber tubing were used to distend the pouches. The size of the balloon was adjusted to the size of the pouch. Similarly, the amount of air introduced into the balloon varied with the various types of pouches. In the animals with subtotal gastric pouches (dogs 1 and 2) approximately 200 cc. of air was used; in the dogs with intra-abdominal pyloric pouches (dogs 3 and 4), about 50 to 100 cc.; and in the animals with subcutaneously transplanted pyloric pouches (dogs 5 and 6), 10 to 20 cc. In each instance the amount of air used induced strong contractions. In most instances the distention period was 30 minutes. Usually the balloon was held in the pouch by hand and a glass hypodermic syringe was kept attached to the rubber tube leading to the balloon. The plunger of the syringe was held in by hand with moderate resistance so that during the height of the contraction wave the plunger was displaced.

Urecholine. In the experiments in which urecholine² (carbamyl beta-methyl choline) was used, 1.5 mgm. of the drug was injected intramuscularly in an oil and beeswax vehicle containing 10 mgm. of the drug per cc.

RESULTS

Basal secretion. All of the animals at times secreted some free HCl during the basal period. This occurred both in the gastric fistula dogs (no. 5, 6) as well as in the animals with transplanted fundic pouches (no. 1-4). However, in all animals free acid was more often absent from than present in the specimens collected during the basal period. The data on the basal secretion are included in the tabular summaries (tables 1 and 2).

Secretion of acid by the pouch which was being distended. In dogs 1 and 2 the main stomach pouch was distended and the secretion was collected from the fundic transplant. Secretion of acid gastric juice by the distended main stomach pouch regularly occurred but no effort was made to collect and measure this juice. Likewise in animals 3 and 4 in which the intra-abdominal pyloric pouch was the one distended, on the few occasions on which tests were made the secretion from the distended pouch was shown to contain free HCl. These pouches thus obviously contained some acid-secreting mucosa in addition to the entire pyloric portion of the stomach. The pyloric pouches of dogs 5 and 6 were fashioned from the same portion of the stomach as those of dogs 3 and 4 but were subcutaneously transplanted. No acid secretion from these subcutaneously transplanted pyloric pouches was ever observed even

² The urecholine was kindly supplied by Dr. D. F. Robertson of Merck and Co.

though it is probable that the line of transection was high enough to have included some acid-secreting cells. The only secretion ever observed from these transplanted pyloric pouches was a few drops of mucoid fluid.

Secretion by the fundic glands in response to distention of the pyloric portion of the stomach. In all of these experiments either the portion from which the secretion was

TABLE 1

DOG	ALL TESTS		POSITIVE TESTS			
	No. of + tests	No. of - tests	Highest pre-distension basal secretion		Response to distension	
			Range	Average	Range	Average
			mgm. HCl per hr.		mgm. HCl per hr.	
1	18	4	0-3.2	1.8	2.4-16.8	11.7
2	4	3	0-1.2	1.2	3.1-14.2	8.9
3	3	4	0 ¹	0	14.6-42.6	32.2
4	4	3	0-16.4	9.9	4.0-32.6	22.4
5	7	9	0-13.5	0.9	1.0-40.0	10.8
6	1	7	—	0	—	6.6
Totals and average	37	30		2.3		15.4

¹ This animal secreted free acid during the basal period during the course of several tests with negative response.

TABLE 2

DOG	BEFORE URECHOLINE						AFTER URECHOLINE					
	No. of Trials	Positive Responses					No. of Trials	Positive Responses				
		No.	Highest Basal		Response			No.	Highest Basal		Response	
			Range	Avg.	Range	Avg.			Range	Avg.	Range	Avg.
		mgm. HCl per hour						mgm. HCl per hour				
3	5	1	—	0	—	42.6	8	8	0-8.2	2.1	6.0-147.2	42.9
4	7	4	0-9.1	5.0	4.0-32.6	21.4	10	9	0-16.0	9.2	12.6-156.8	74.2
5	8	6	0-6.3	1.2	1.0-40.0	6.2	10	10	0-32.0	14.3	15.2-212.0	89.8
6	7	1	—	0	—	6.6	10	9	0	0	2.0-186.8	41.6
Total	27	12					38	36				

collected (*Group I*, dogs 1-4) or the portion being stimulated by distention (*Group II*, dogs 5, 6) was subcutaneously transplanted.

Frequency of positive response. The response to distention was considered to be positive if the secretion of free HCl during the half-hour period of distention or the immediately ensuing half-hour period was higher than any of the basal half-hour periods for that day. On this basis positive responses occurred in 29 of 43 trials in the dogs in *Group I* and in 8 out of 24 trials in the dogs in *Group II*. Table 1 summarizes the results of these experiments and figure 3 is a graphic record of one such experiment in dog 1.

Latency and duration of responses. Frequently a drop of Töpfer's solution was added to the collection flask during the period of distention in experiments in which there was no free hydrochloric acid during the basal period. In this way the time of onset of free acid secretion could be noted. There was considerable variation in the latent period between the beginning of distention of the pyloric portion and the appearance of free hydrochloric acid in the juice from the fundic glands. The shortest latent period noted was about 10 minutes. However, a few times no free hydrochloric acid appeared during the half-hour distention period and the response to distention occurred only in the half-hour period following the cessation of distention.

The rate of secretion usually had returned to the basal level by the second half hour after cessation of distention. Occasionally the response lasted only during the

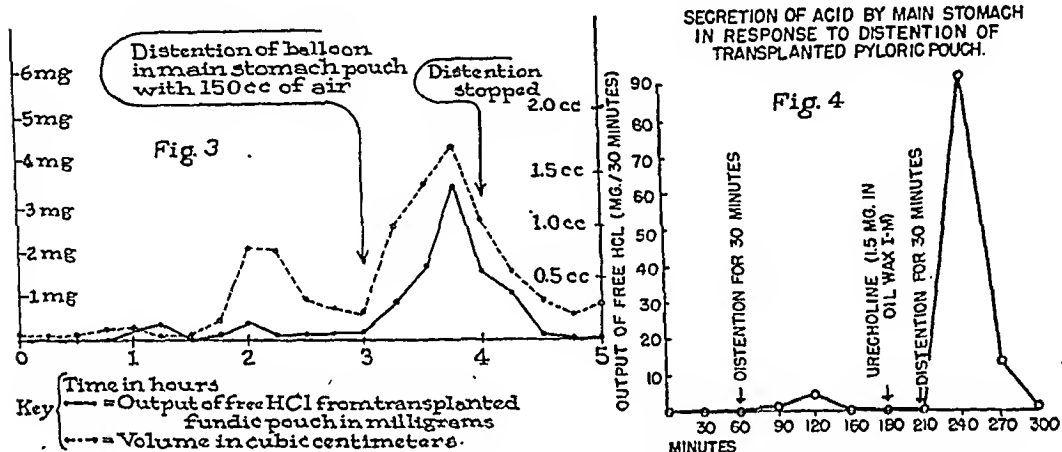


Fig. 3. GRAPHIC RECORD of an experiment in a dog with a transplanted fundic pouch and a pouch of the remainder of the stomach showing acid secretory response of transplanted pouch to a distention stimulus applied to the main stomach pouch.

Fig. 4. GRAPHIC RECORD of the results of an experiment in a dog with a transplanted pyloric pouch and a fistula of the main stomach. The response of the main stomach to distention of the transplanted pyloric pouch was very small before injection of urecholine. Urecholine alone did not stimulate gastric secretion, but it greatly potentiated the secretory response to subsequent distention of the pyloric pouch.

period of distention whereas on a few occasions the response appeared to persist for as long as one hour after the cessation of distention.

Urecholine. Because in some dogs the frequency with which positive responses occurred was quite low, some method of potentiating the response was sought. The work of Gray and Ivy with mecholyl (13) had suggested that parasympathomimetic drugs potentiate the response to stimuli such as histamine. Our more recent studies (14) have confirmed this finding and have shown that the distention stimulus is also potentiated by these drugs.

A series of experiments was therefore performed in which urecholine was used. Dogs 3, 4, 5 and 6 were used. A control distention without urecholine was first performed. One-half hour after this first distention, when the response to it, if any, had subsided, 1.5 or 3.0 mgm. of urecholine in oil and wax was injected intramuscularly. One hour after the urecholine injection a second distention was performed. In a few

of the experiments the preliminary distention before urecholine injection was not carried out. The results are summarized in table 2.

It will be noted that the response to urecholine alone varied from zero to a rather high value. However, even when a secretory response did not occur in response to the urecholine alone the response to distention was enhanced by the urecholine. This is illustrated by the graphic record of an experiment in dog 6 (fig. 4).

Positive responses occurred in 12 out of 27 experiments in which distention was performed before urecholine was administered, whereas 36 out of 38 trials were positive after the drug.

DISCUSSION

When Gregory and Ivy (4) performed experiments essentially similar to our experiments on dogs of *Group I*, they failed to observe a secretory response in the transplanted fundic pouch when the main stomach pouch was distended. In several such 'two-pouch dogs' other than those which were used in the present studies we too were unable to elicit such a response. A number of factors may have contributed to the failure of these earlier experiments. As already indicated we believe that the most important of these factors is the responsiveness of the transplanted pouch preparation, and this has been enhanced chiefly by making the transplanted pouches larger.

All of the transplanted fundic pouches used in the present study secreted free hydrochloric acid at times during the basal period. This did not occur in Gregory and Ivy's animals nor has it been observed in other transplanted fundic pouches previously studied in our laboratories. Furthermore, the responses of the transplanted pouches used in the present studies is definitely greater than that which has been observed in previous studies.

Another factor which must be taken into consideration is the amount of distention used as a stimulus. If the pouch which is being distended has intact sympathetic innervation, excessive distention may lead to retching and vomiting and we (15) have shown that retching inhibits histamine stimulated gastric secretion even in the transplanted pouch of the fundic portion of the stomach. Chang and Lim (10) studying the effect of distention of the isolated pyloric pouch (sympathetics intact) upon acid secretion by the fundic glands stated that "volumes of air larger than 20 cc. not infrequently precipitated nausea and vomiting, and usually failed to excite secretion from the fundus." The statement of Gregory and Ivy (4) is of interest in this regard: "even when excessive pressures are used (15-30 cm. H₂O), causing signs of nausea in the animal (salivation, restlessness, retching), and are maintained for periods up to 2 hours, *no production of free acid from the transplant is detected*, [italics theirs] despite the fact that the amount of free acid secreted meanwhile by the main pouch may be considerably in excess of that evoked by perfusion with liver extracts, which has been shown to cause a humoral response from the transplant." In the present studies we have attempted to avoid the occurrence of nausea by using only moderate distention. In the dogs in *Group II*, the pyloric pouch was subcutaneously transplanted and nausea did not occur even with severe distention.

The mechanical and chemical stimuli for gastric secretion had previously been

shown to be similar in a number of ways: *a*) both chemical and mechanical stimuli are more effective in stimulating acid secretion by the fundic glands when they act in the pyloric region than when they act in the fundic region, *b*) both are prevented from acting by the application of procaine to the part of the stomach being stimulated and *c*) the action of both is blocked by atropine. With the present studies the only apparent discrepancy in the mechanism of action of the two stimuli for the gastric phase of gastric secretion is resolved by demonstrating that the mechanical stimulus, like the chemical stimulus, releases a humoral agent. Inasmuch as the question of absorption of the stimulating agent does not arise in the consideration of the nature of the humoral agent for the distention stimulus, the conclusion that it is hormonal in nature would appear to be justified.

The present studies give no new information about the nature of the hormonal agent.

SUMMARY

Distention of the pyloric portion of the stomach stimulates the secretion of hydrochloric acid by the fundic glands. This effect still occurs when all nervous connections between the stimulated portion of the stomach and the portion responding with secretion have been interrupted. This interruption can be accomplished by subcutaneous transplantation of either the part of the stomach which is to be stimulated, namely the pyloric portion, or the part which responds to the stimulus, the fundic portion. This demonstration of the humoral transmission of the distention stimulus is considered to constitute conclusive evidence for the existence of a hormone for gastric secretion.

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EFFECT OF HYPOXIA UPON TEMPERATURE REGULATION OF MICE, DOGS, AND MAN¹

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IN SETTING up controls for study (12) of the rôle of the adrenal cortex on resistance of mice to hypoxia, it became apparent that temperature effects at times dominated the picture. The further study of these effects is here recorded.

METHODS

Mice. Mice were exposed in individual compartments of decompression chambers at temperatures constant to $\pm 0.25^{\circ}\text{C}$. and pressures controlled to ± 2 mm. Hg. Body temperatures were measured by copper-constantan thermocouples in the rectum. Mice, fasted 4-6 hours, were placed in the decompression chambers for 30 minutes to allow temperature equilibrium and then the pressure was reduced gradually, critical levels being reached in from 90-120 minutes. Death, as determined by the last respiration, was the endpoint, and the atmospheric pressure or equivalent altitude (3) at which this occurred was considered to be the hypoxic ceiling for the animal in question.

Dogs. Dogs breathed low-oxygen gas mixtures flowing through a mask at a rate which prevented significant rebreathing. Rectal and brain temperatures were recorded using copper-constantan thermocouples. The brain thermocouple was inserted into the parietal area, several days before the hypoxic test and the leads brought out through the skin. At the time of the test, the leads were soldered to the potentiometer.

Humans. Human subjects were exposed to hypoxia by breathing gas mixtures while sitting in a cool room. A standard uniform of light underwear, socks, mitts and hood covered all parts of the body except the face. Temperatures were recorded from the rectum by thermocouple and from the skin of the forehead, arm, thigh, calf, ankle and three points on the trunk by thermocouple and/or radiation pyrometer. Subjects breathed through a face mask connected to an 87-liter closed system by a valved circuit. Carbon dioxide was absorbed with soda lime. Respiratory air flow, respiratory rate and oxygen consumption were recorded. Oxygen could be added to the system at controlled rates to maintain or change the content as desired.

RESULTS

Mice. As determined on 54 mice, hypoxia (250 mm. Hg pressure or less) causes mice to become almost completely poikilothermic; between 4° and 24°C . rectal temperatures of mice exposed to hypoxia as described were on the average less than

Received for publication July 10, 1947.

¹ Parts of the work reported here were conducted under contract with the Committee on Medical Research, Office of Scientific Research and Development (OEMCMR43) and with the Army Quartermaster Board (QMB T-158).

1°C. above the temperature of the environment. Seventy-eight controls similarly ventilated at atmospheric pressure in this range of temperature maintained more

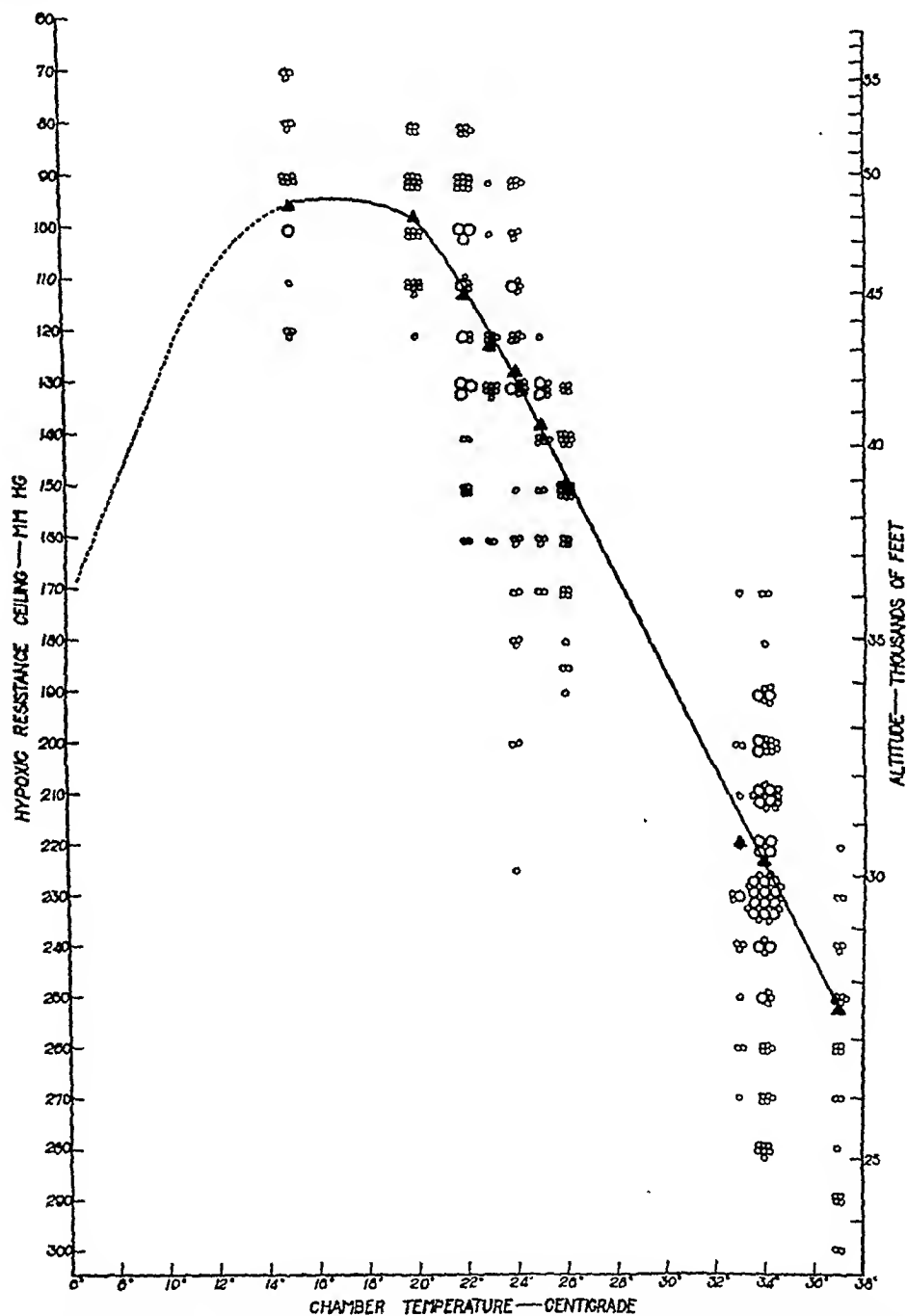


Fig. 1. EFFECT OF ENVIRONMENTAL TEMPERATURE on the survival of mice during progressive lowering of atmospheric pressure. The small circles indicate the hypoxic ceiling of individual mice. The large circles indicate the hypoxic ceilings of 10 mice dying with identical ceilings. The triangles show the mean for each temperature group.

variable body temperatures which were 8°–15°C. higher than the environment. Voluntary muscular activity and shivering seen at normal oxygen tension were not

seen in the hypoxic mice although they occasionally carried out coordinated purposeful movements.

The effect of environmental temperature on the resistance to acute hypoxia was studied on 824 mice over the temperature range from 15°C. to 37°C. (fig. 1). At an environmental temperature of 34° mice maintain a body temperature of approximately 37°C. At this temperature the range of lethal ceiling for mice exposed to acute hypoxia was 25,000-36,000 feet (280-170 mm. Hg) with a mean ceiling at 30,000 feet. Decreasing the chamber temperature from 34°C. to 20°C. increased the

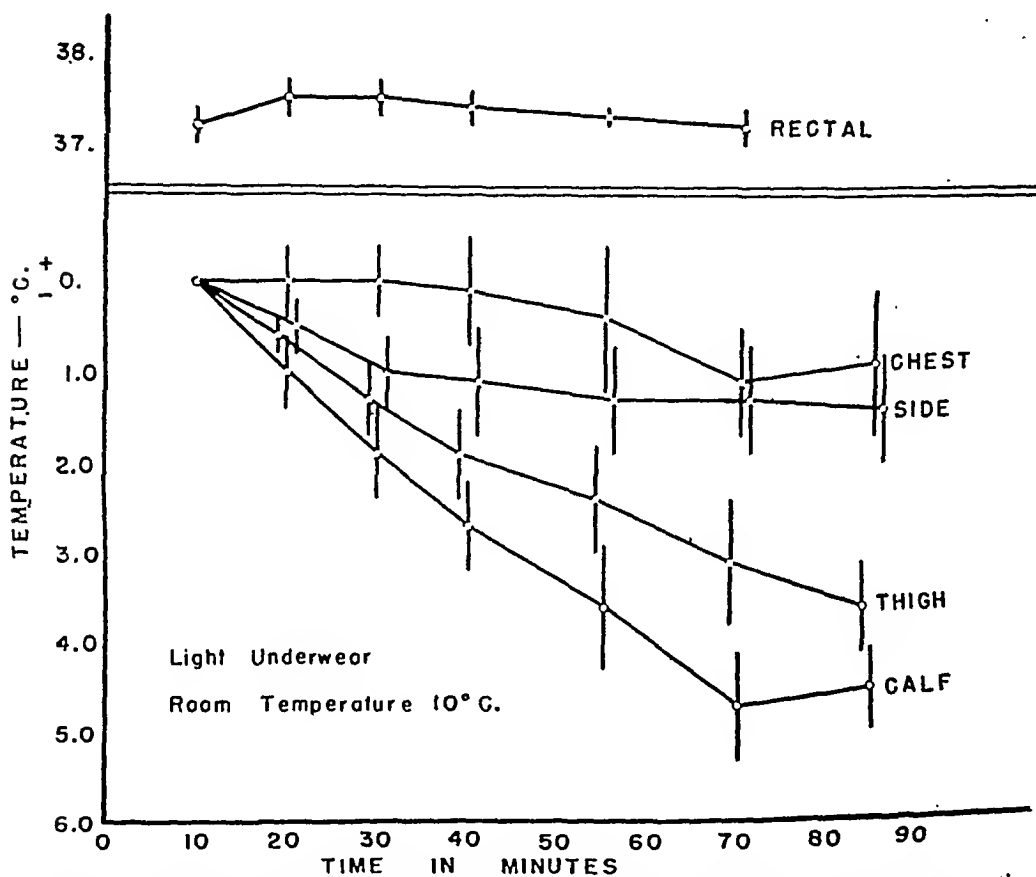


Fig. 2. EFFECT OF COLD ON RECTAL AND SKIN TEMPERATURES IN MAN. Sixteen observations on four subjects. Vertical lines indicate standard deviation of individual temperature readings from the mean.

hypoxic ceiling to 43,000-54,000 feet (120-70 mm. Hg). As the temperature decreased below 15°C., the resistance to hypoxia decreased.

Dogs. In five experiments in which dogs were exposed to hypoxia at environmental temperatures of 12°-25°C., the body temperature regularly declined as the oxygen content of the inspired air fell below 10 per cent until at 6 per cent the average fall was 1.34°C. for rectal and 1.33°C. for brain temperatures; the falls were not less than 1.0°C. and they occurred within 30 to 60 minutes after reaching 10 per cent oxygen. Controls breathing normal air showed at the end of three hours a fall in rectal temperature of less than 0.25°C. Shivering which occurred in controls in normal air was regularly inhibited in the hypoxic group when the oxygen content

became less than 10 per cent but was established promptly and markedly on terminal admission of room air.

Man. Figure 2 shows the temperatures of healthy young men exposed as described to a room temperature of 10°C . in normal air. The greatest fall in rectal temperature was 0.2°C . Oxygen consumption rose an average of 38 per cent after the onset of shivering which in all cases began within 50 minutes of exposure. In contrast to these controls are the temperature changes accompanying hypoxia in

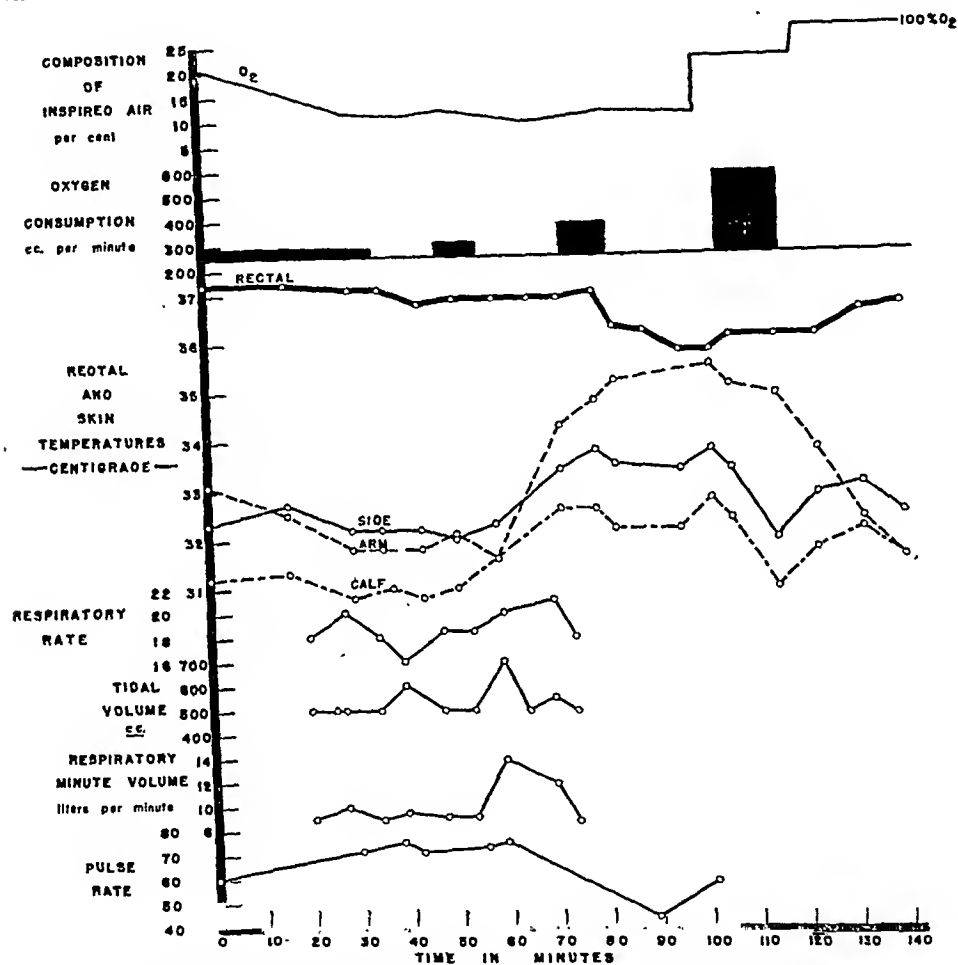


Fig. 3. EFFECT OF PROGRESSIVE LOWERING OF OXYGEN CONTENT in the inspired air of man on oxygen consumption, and on rectal and skin temperatures. Room temperature 19°C .

environmental temperatures of 11° to 22°C . Figures 3 and 4 represent the protocols of two experiments which are representative of four of five experiments. In all four shivering was inhibited during hypoxia and the oxygen consumption rose not at all or to a distinctly lesser degree than in controls (av. 12 per cent); in addition there was evidence of increased heat loss as exhibited by skin temperatures increased above controls. The result was a striking drop in rectal temperature (av. 1.04°C .). On readmission of normal air, violent shivering began, oxygen consumption rose, skin temperatures fell and rectal temperature rose. In a fifth experiment, shivering was not inhibited and although skin temperatures rose there was no fall of rectal temperature.

DISCUSSION

In three species, hypoxia is seen to be accompanied by loss of temperature control. Inhibition of shivering or of voluntary activity appears to be an important

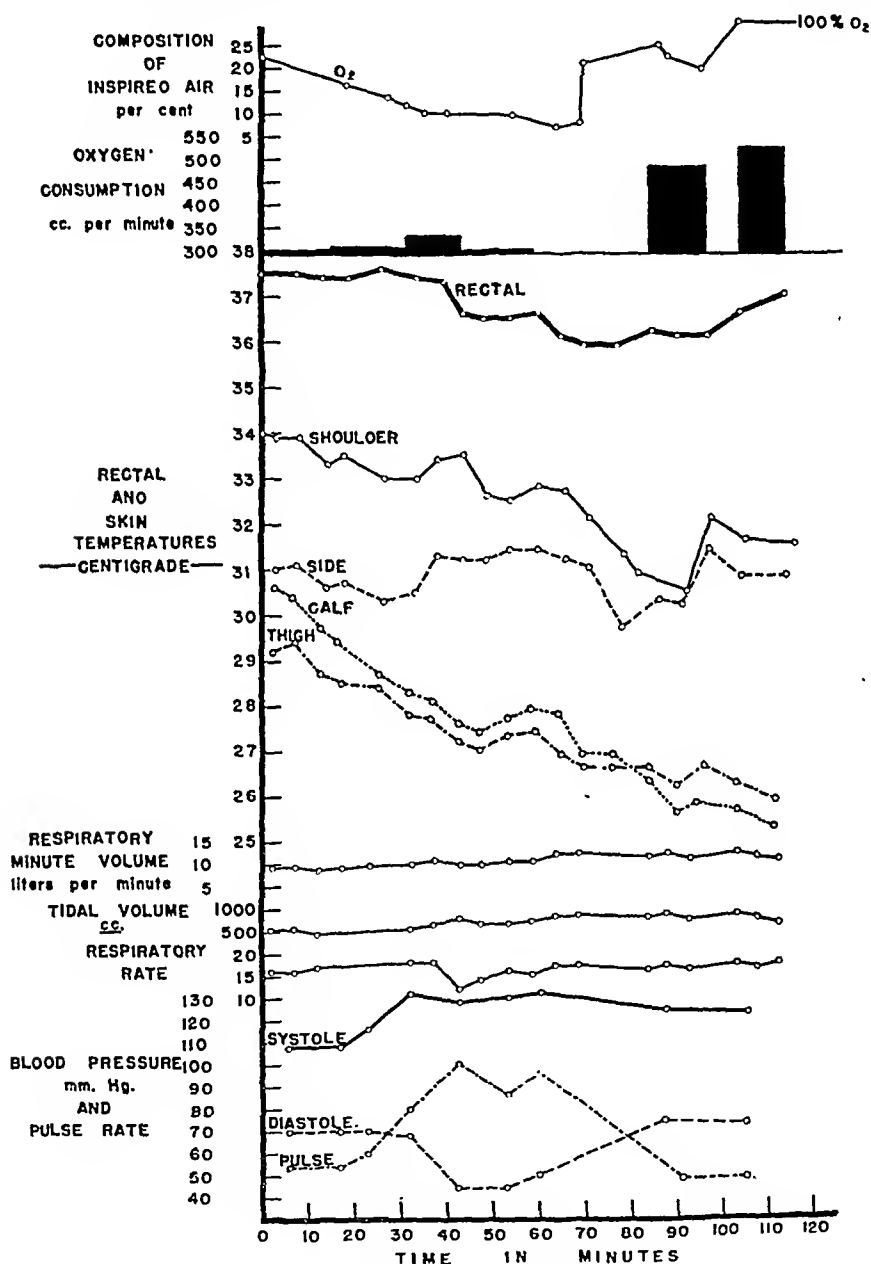


Fig. 4. EFFECT OF PROGRESSIVE LOWERING OF OXYGEN CONTENT in the inspired air of man on oxygen consumption and on rectal and skin temperatures. The spread in pulse pressure was accompanied by the appearance of a capillary pulse. Room temperature 11°C.

effect. It should be noted however that in one of the human experiments and in one of the dog experiments, the fall in temperature during hypoxia occurred at environmental temperatures above those which induced any noticeable shivering under

control conditions. Peripheral vasodilatation also plays a rôle; not only did the skin temperatures of hypoxic man stay above those of controls, but peripheral pulses and increased pulse pressures were noted. In independent unpublished experiments, increased peripheral flow to the lower leg was demonstrated in man during hypoxia by direct plethysmography.

There have been numerous reports of loss of homeothermy in laboratory animals during exposure to hypoxia (1, 2, 4-10, 13, 14, 15). The striking protection against death at altitude which is afforded by drop in the body temperature of mice would seem to be due to the decreased oxygen demand at lower body temperatures and has a parallel in the increased altitude tolerance of thyroidectomized animals (16). In man, however, a decrease in body temperature is associated with decreased mental ability (11) and would, therefore, not be desirable in situations where he must think and act.

SUMMARY

Hypoxia decreases the ability of mice, dogs and men to control body temperature during exposure to cold. The loss of temperature control and fall in body temperature is most marked in the mouse and least marked in man. Hypoxia inhibits shivering in all three species studied. In man exposed to cold the suppression of an increased oxygen consumption by the hypoxia was demonstrated. In addition to this effect of hypoxia on heat generation it was also shown for man that hypoxia results in a greater dissipation of heat from the skin in a cold environment. Decreased environmental and body temperature favored survival of mice exposed to progressive hypoxia; between 37°C. and 20°C., the survival ceiling increased approximately 1000 feet per 1°C. drop in temperature.

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ADRENAL CORTEX AND ALTITUDE TOLERANCE¹

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THE possible importance of the adrenal cortex in the bodily responses to hypoxia has been inferred from a variety of observations (1, 4, 5, 6, 7, 8, 11, 12, 15, 16). The present work undertaken in connection with problems in war-time aviation records the effects of adrenal cortical compounds on the resistance of normal animals to acute hypoxia.

METHODS

Mice. Albino mice weighing 17-22 grams were exposed to hypoxia in low-pressure chambers as described previously (10). Unless otherwise specified, the mice were fasted four to six hours prior to exposure to hypoxia.

The decompression chambers in the majority of the experiments were maintained at approximately 34° C. which permits mice to maintain body temperatures comparable to that of man. The pressure levels are also referred to in terms of equivalent altitude (2).

Dogs. Medium-sized dogs were exposed in a well ventilated chamber to progressively increasing hypoxia, approximately equal to an ascent of 1000 feet per minute to critical levels and then progressively more slowly to physiological ceiling, which was reached in about two hours. The dogs had previously been satisfactorily conditioned to respond to light or sound stimuli by moving across a low barrier from one end of the decompression chamber to the other. The auditory stimulus was a tone of 4096 cycles per second continuously variable from 0 to 95 decibels, and the visual stimulus was a variation of light intensity within the chamber from 47 to 16 footcandles in stages. At atmospheric pressure a sound stimulus of 55 decibels or a change of light intensity of seven per cent consistently initiated the desired response. Motor response of the dogs was evaluated in two ways: 1) slowness of response and 2) motor incoordination estimated in three grades. When the animals were prostrate in the hypoxic test, attempts to rise were interpreted as response to sound or light stimuli.

RESULTS

Tests of the effects of adrenal cortical compounds on resistance to acute hypoxia were made on 1038 mice. The principal results are shown in table 1.

Received for publication July 10, 1947.

¹ This work was originated in correspondence with the Committee on Aviation Medicine, and conducted under contract (OEMCMR 43) with the Committee on Medical Research, Office of Scientific Research and Development. The authors wish to thank Dr. E. C. Kendall for his generosity in supplying them with extracts.

Kendall's aqueous adrenal cortical extract in mice (1 cc. = 75 grams of gland) in doses of 0.25 cc. or more was clearly effective (experiments 7-8) in raising ceiling in all instances. Attention is drawn to the quite adequate numbers of animals used. This contrasts with the negative results obtained in experiments using other cortical

TABLE 1. EFFECT OF INJECTED MATERIALS ON ABILITY OF MICE TO SURVIVE IN ACUTE HYPOXIC TEST

Treatment	Time of Injection hrs. before test	No. Mice	Chamber Temp.	Lethal Ceiling		p ¹
				Control	Treated	
			°C	mm. Hg		
1. Wilson adrenal cortical extract (aqueous, 75 gm. gland/cc.) 0.5-1.0 cc.	4	128	22	113 ± 2.3	107 ± 2.3	.072
2. Wilson A.C.E. (same) 0.5-1.0 cc.	1	56	35	224 ± 4.5	231 ± 5.2	.317
3. Wilson A.C.E. 0.25-1.0 cc.	1	96	34	213 ± 2.3	220 ± 2.1	.05
4. Wilson A.C.E. 2.0 cc.	1	24	34	233 ± 5.0	224 ± 4.0	.174
5. Upjohn A.C.E. (aqueous 40 gm. gland/cc.) 0.5-1.0 cc.	0.5	32	22	115 ± 3.7	121 ± 4.0	.368
6. Upjohn A.C.E. (alcohol removed by vacuum distillation) 0.5-1.0 cc.	3	56	25	139 ± 2.9	140 ± 3.1	.990
7. Kendall's A.C.E. (aqueous 75 gm/cc.) 0.25 cc.	0.5	214	34	223 ± 1.8	209 ± 1.7	<.001
8. Kendall's A.C.E. (aqueous) 0.5-1.0 cc.	4	32	35	239 ± 4.9	225 ± 2.0	.008
9. Kendall's A.C.E. (aqueous) 0.1 cc.	0.5	32	34	225 ± 4.5	218 ± 4.5	.230
10. Kendall's A.C.E. (oil 150 gm/cc.) 0.5-1.0 cc.	4	32	34	214 ± 4.0	214 ± 2.6	1.0
11. D.O.C.A. in oil, 0.01 mgm.	6	32	25	142 ± 4.0	142 ± 3.5	1.0
12. 5 per cent glucose, 1.0 cc. intraperitoneally	1	32	34	227 ± 1.8	217 ± 2.3	<.001
13. Kendall's A.C.E., 0.25 cc. + 1 cc. 5% glucose intraperitoneally	1	64	34	227 ± 2.7	213 ± 3.1	<.001

¹ Probability that the variation from the control is due to chance in random sampling.

material including Kendall's extract in oil (1 cc. = 150 grams of gland). In further examinations of the positive results with Kendall's aqueous extract, it was found by the use of rectal thermocouples that the temperature of injected mice was 1.5°C. higher during hypoxic test than that of controls. The blood glucose level also was 11 mgm. per cent higher than in controls and in separate experiments injected glucose (experiment 12), resulting in a mean increase of the blood sugar level of 70 mgm. per

cent, gave an improved tolerance approaching that produced by Kendall's extract. Mice injected with glucose and Kendall's extract did not show a greater resistance to hypoxia than those injected only with Kendall's extract (experiment 13).

The possibility of the positive results being due to epinephrine was considered. In eight experiments involving 240 animals, it was found that epinephrine alone or in combination with cortical extracts did not increase hypoxic resistance, and when the dose was 2.0 micrograms or more per animal the resistance was lowered.

Adrenal size. Studies of the adrenal weights of 57 normal mice showed no relation to their resistance to acute hypoxia. Thirty-one mice exposed to an air pressure of 250 mm. Hg for 25 days showed adrenal weights increased to 20.2 ± 0.8 mgm./100 grams body weight as compared to 12.8 ± 0.6 mgm. for controls, without however exhibiting altered resistance to hypoxia. In view of the adrenal atrophy reported following injections of natural or synthetic adrenal cortical materials (9, 13), mice were so treated for as long as 13 days with daily doses of either 5 mgm. D.O.C.A. per

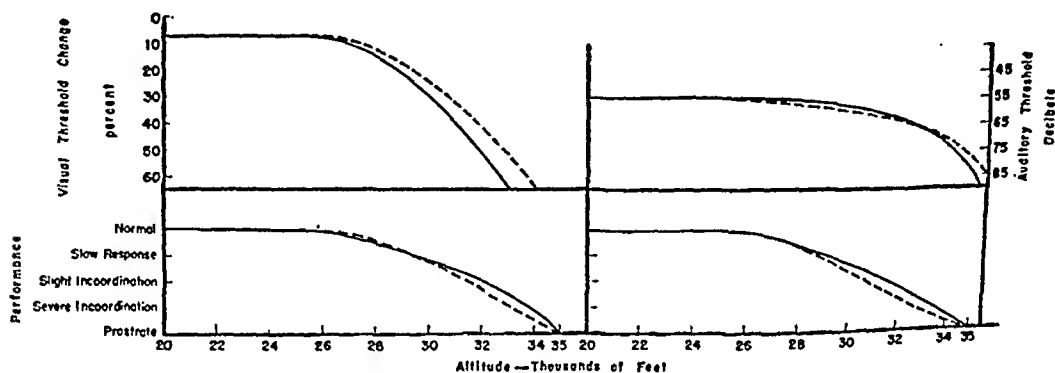


Fig. 1. EFFECT OF ADRENAL CORTICAL EXTRACT (1 cc. = 150 gm. adrenal gland) on motor performance and on auditory and visual discrimination in conditioned dogs during exposure to acute hypoxia (see METHODS in text). ——— controls, ——— dogs injected subcutaneously with 50-125 cc. of adrenal cortical extract one to four hours prior to exposure.

100 grams body weight or aqueous extract representing adrenal glands in excess of the animals weight. Such treatment had no effect on resistance to acute hypoxia.

Dogs. The results are shown in figure 1. The responses of the animals were satisfactorily uniform in repeated tests. It will be seen that adrenal cortical materials were without effect on motor performance or on visual or auditory discrimination.

DISCUSSION

The positive results with Kendall's extract agree with those reported by Thorn, Clinton, Davis, and Lewis (16) who found that it increased the resistance of rats to acute hypoxia although neither desoxycorticosterone acetate nor Compound E had this protective effect. They used a different type of exposure, but the magnitude of effect was approximately the same as reported in this paper. The only other report (8) of improved altitude tolerance with cortical material carries with it the objection that a charcoal absorbate was administered orally, and charcoal alone has been shown

(3) to give protection at altitude. It should be pointed out that Kendall's extract did not produce super-animals in respect to altitude tolerance, in that its effect was to move the average tolerance up toward the maximum, but not to raise the ceilings of individual animals above that which was observed in a small fraction of uninjected controls. Since the rectal temperature of the animals injected with Kendall's extract was approximately 1.5°C . higher than that of uninjected controls and since altitude tolerance decreases by 1500 feet as rectal temperature increases this amount (10), it is possible that the effect is somewhat greater than that represented by a 1500-foot elevation of ceiling.

The experiments with dogs had the advantage over those with the mice in that, in place of the crude criterion of death, tests of higher function were used. The doses of Kendall's extract were large (equivalent to 990 grams whole adrenal/kgm. body weight) and on a body-weight basis slightly in excess of the effective dose of Kendall's extract for mice. This dose exceeds the total daily adrenal cortical secretion of the normal dog as estimated by Vogt (17).

The central nervous system, which is of first importance for survival at low oxygen tension and which burns carbohydrate almost exclusively, tolerates poorly drops in oxygen or glucose supply. It is interesting, therefore, that lowered body temperature (10) and thyroid deficiency (14), both of which reduce the demands of the tissues for oxygen, have both been seen to be accompanied by improved resistance to hypoxia. Equally interesting is the possibility that the beneficial effect of Kendall's extract is due to its gluconeogenetic potency. Against this possibility is the non-effectiveness of Compound E of Kendall as observed by Thorn, Clinton, Davis and Lewis (16).

SUMMARY

The effect of injected adrenal cortical compounds on the resistance of normal mice to acute hypoxia was studied. Two commercial aqueous adrenal cortical extracts and also desoxycorticosterone acetate injected subcutaneously in large doses were without beneficial effect. An aqueous extract prepared by Kendall when injected in doses greater than 0.25 cc. per mouse increased the mean hypoxic ceiling 1500 feet above that of uninjected normal mice; glucose injected intraperitoneally increased the resistance to hypoxia nearly as much as Kendall's extract. Pretreatments of mice calculated to induce hyper- and hypo-function of the adrenal cortex were without effect on altitude tolerance as judged in subsequent acute tests, and no correlation was found between hypoxic resistance and induced or spontaneous variations in adrenal size. Large doses of Kendall's extract were without effect on the deterioration of coordination, vision and hearing of dogs exposed to acute hypoxia.

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ANTI-HORMONE PROPERTIES OF UREA-DENATURED SHEEP GONADOTROPIN

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BY MEASURING the rates of inactivation, it has been demonstrated that the denaturation of chorionic gonadotropin, either by heat in aqueous solution or by 40 per cent aqueous urea solution, results in a series of reaction products possessing biologic activity (1). At 99° C. in aqueous solution, the rate of destruction of the original hormone is immeasurably fast. However, the reaction product, which retains 5 per cent of the original biologic activity, loses only half of its activity when similarly heated for 30 minutes. As reported in this paper, comparable results with urea denaturation have been obtained with the sheep pituitary gonadotropin. It was postulated (2) that the biologic activity of the protein hormones is dependent, not so much upon structure in the sense of the atomic linkage theory, but upon the spacing of active adsorption points due to secondary valence effects. A derangement of spacing occurs in denaturation and in any chemical reaction which changes the adsorption foci directly or indirectly by the weakening of hydrogen bonds.

The question, therefore, arose as to whether a denatured hormone which retained biologic activity as measured by some arbitrary physiologic response would retain the same degree of activity when measured by some other physiologic response. In the experiments recorded in this paper, the sheep pituitary gonadotropin, which has been denatured by urea to retain 8 per cent of its original gonadotropic activity, has been assayed by two other widely different responses, viz., the antagonism and the anti-hormone phenomena. The problem seeks to answer whether the multiple effects manifested by the gonadotropins are dependent upon multiple hormones (the dual hormone theory), or whether multiple effects may be attributed to spatial arrangements of active points in a single protein hormone molecule. Chorionic gonadotropin, which exhibits nonspecific augmentation under certain conditions (3, 4), and mare serum gonadotropin, which is both follicle and interstitial cell-stimulating, have both been purified to a degree indicating that the properties are incorporated in a homogeneous protein (5, 6). By fractionation of the sheep gonadotropin, however, a homogeneous protein has been isolated which is claimed to be solely interstitial cell-stimulating (7); another fraction which is follicle-stimulating has not been obtained in pure form. In our experience this fraction is also interstitial cell-stimulating and manifests the antagonism and nonspecific augmentation phenomena (8). As Cole (9) has pointed out, evidence has not been observed for the actual secretion by the pituitary of interstitial cell-stimulating hormone devoid of the other properties. From the observations cited in the foregoing discussion, the possibility arises that the so-called interstitial-cell-stimulating hormone may be nothing other than a denaturation variant of the intact sheep pituitary gonadotropin, a precursor or a dissociation fragment. In the present study the unfractionated sheep pituitary extract is used to obviate criticism of original denaturation or fragmentation by dissociation. Its properties have been described (10).

Received for publication March 24, 1948.

EXPERIMENTAL

Gonadotropic effect. The assay curve for the gonadotropin measured by increase in ovarian weight in the immature rat under conditions assuring maximum response is given in an earlier paper (10). Survival of less than 25 per cent of the original activity does not produce a significant increase in ovarian weight. In this range, uterine weight increase lends itself to a very sensitive assay curve. The gonadotropin subjected to 6 hours' treatment in 40 per cent urea concentration at 37.5° C. retained 7 ± 1.5 per cent of its original activity (10). In the present experiments, the time of reaction was extended to 8 hours in two experiments and to 24 hours in a third. At 8 hours 1.0 mgm. denatured hormone produced a 56 ± 10 mgm. uterine weight, compared with a 27 ± 1.0 mgm. uterine weight for .05 mgm. of intact hormone, giving a recovery of 7 ± 1.5 per cent. In a duplicate experiment the uterine weight for 1.0 mgm. denatured hormone was 71 ± 13 mgm., compared with 92 ± 9 mgm. for 0.10 mgm. of intact hormone, giving a recovery of 8 ± 1.5 per cent. These experiments show that after six hours' treatment, an additional two hours in 40 per cent urea solution produces no further measurable destruction of gonadotropic activity. In the first two hours of treatment the hormone loses 70 per cent of its gonadotropic activity (10). Data for 24 hours' treatment by the antagonist assay procedure showed a recovery of 5 ± 1.0 per cent.

Antagonism phenomenon. 1.0 mgm. of the pituitary hormone when injected subcutaneously with 0.5 mgm. Cu at pH 8.0 produces a 100 mgm. increase in ovarian weight in the immature rat. In groups of eight rats the standard deviation of the mean is very nearly 10 mgm. In the assay procedure, 1.0 mgm. of hormone is contained in one cc. volume and is administered 0.25 cc. per day for four days. Autopsy is performed 96 hours after initial dosage. Figure 1 illustrates the effect of the simultaneous administration of the same hormone, in isotonic saline solution and without copper, when given intraperitoneally. The total dose (0.1, 0.2, 0.5 or 1.0 mgm.) was contained in one cc. volume and was given 0.25 cc. per day for four days immediately after the respective subcutaneous injections. It will be noted that the sensitive portion of the curve lies between 0.1 and 0.2 mgm. The data in the chart are the mean values for 8 to 10 rats, and in all cases a control with litter mates was run simultaneously for one mgm. hormone with 0.5 mgm. Cu given subcutaneously. The augmentation produced by the control was set at 100 per cent, since the experimental variation was close to one standard deviation of the mean. The assay curve given in figure 1 is a refinement of semiquantitative data previously reported (11).

Assay of the denatured hormone for antagonism. Three assays were performed, two for preparations subjected to 40 per cent urea solution for eight hours at 37.5° C. and one for preparations subjected to 40 per cent urea solution for 24 hours at 37.5° C. For the 8-hour samples, one mgm. showed an augmentation of 103 ± 13 per cent and two mgm. showed an augmentation of 35 ± 3 per cent. Taking three times the standard deviation of the mean as the limit of probability, the assay falls between the limits of 7 and 15 per cent, viz., 11 ± 1.5 per cent. For the 24-hour sample, 2 mgm. showed an augmentation of 82 ± 8 per cent. Taking three times the standard deviation of the mean as the limit of probability, the assay falls between three and eight per cent, viz., 5 ± 1 per cent.

Antihormone effect. Demonstration of the antihormone action in mice is given in the experiments summarized in table 1. The mean weight of the ovary of the three months' old Marsh-Buffalo mouse is 10 mgm. The subcutaneous administration of 0.6 mgm. intact sheep gonadotropin in saline solution over a six-day period produces no increase in ovarian weight as shown in *experiment A₁*. The same amount of hormone given as a copper precipitate at pH 7.5 produces a 13 mgm. increase in ovarian weight (see *experiment B₁* and *experiment G*). This phenomenon is well established and illustrates the effect of delayed resorption to produce a maximal response. In *experiment A₂*, the intact hormone in saline was administered to mice two months of age. At the age of three months, the intact hormone was readmin-

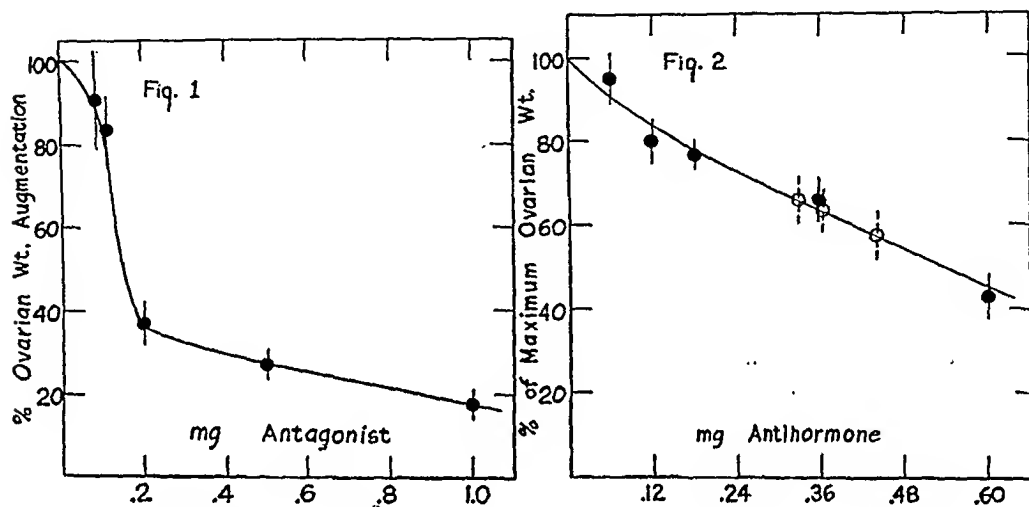


Fig. 1. ANTAGONISM PHENOMENON: The influence of graded doses of hormone given intraperitoneally in reducing the augmentation produced by one mgm. given subcutaneously with copper to delay resorption. Immature rats are used as the test object. The vertical lines through the circles are the standard deviations of the mean.

Fig. 2. ANTIHORMONE PHENOMENON: the influence of graded doses of hormone given at the age of two months upon the gonadotropic effect of the same hormone given at the age of three months. Marsh-Buffalo mice are used as the test object. The black circles represent the response to graded doses of intact hormone as indicated by the abscissa axis. The open circles are the values obtained for 0.6 mgm. of denatured hormone. The vertical lines through the circles are the standard deviations of the mean.

istered as the copper combination. No increase in ovarian weight occurred, the injection at two months exerting a complete antihormone action. A pronounced antihormone effect is also produced when the intact hormone is injected as the copper combination at the age of two months, and the dosage is repeated in the same manner one month later (see *experiment B₂*).

Experiments C₁, C₂, D₁ and D₂ are a repetition of experiments *A₁, A₂, B₁ and B₂*, substituting urea-denatured hormone for intact hormone. In *experiment C₁*, the denatured hormone administered in saline produces no increase in ovarian weight. In *experiment D₁*, the denatured hormone administered with copper produces no ovarian weight increase, showing that the hormone has lost its gonadotropic effect.

In experiments C_2 and D_2 , the denatured hormone either with or without copper is administered to mice two months of age. A month later the intact hormone is administered with copper. In each case a pronounced antihormone effect is noted.

In summary, these experiments show that: a) an antihormone effect is produced whether the hormone is rapidly or slowly resorbed and b) urea denaturation, which completely abolished the gonadotropic effect, did not abolish the antihormone effect. A comparison of experiments A_2 and C_2 suggested that while most of the antihormone

TABLE 1. ANTIHORMONE EFFECT OF SHEEP PITUITARY GONADOTROPIN UPON OVARIES OF MATURE MICE

EXP. NO.	TREATMENT (SERIES, 1 DOSE PER DAY, 6 DAYS)		OVARIAN WT. IN MGM.	DATE KILLED
	1st dose series age 2 months	2nd dose series age 3 months		
A_1	0.6 mgm. intact hormone	—	9 ± 0.9 (8)	2 mo. 8 da.
A_2	0.6 mgm. intact hormone	0.6 mgm. intact hormone with 0.3 mgm. Cu	10 ± 1.2 (8)	3 mo. 8 da.
B_1	0.6 mgm. intact hormone with 0.3 mgm. Cu	—	23 ± 1.8 (6)	2 mo. 8 da.
B_2	0.6 mgm. intact hormone 0.3 mgm. Cu	0.6 mgm. intact hormone with 0.3 mgm. Cu	13 ± 1.0 (7)	3 mo. 8 da.
C_1	0.6 mgm. denatured hormone	—	7 ± 0.2 (8)	2 mo. 8 da.
C_2	0.6 mgm. denatured hormone	0.6 mgm. intact hormone with 0.3 mgm. Cu	13 ± 1.3 (8)	3 mo. 8 da.
D_1	0.6 mgm. denatured hormone with 0.3 mgm. Cu	—	7 ± 0.6 (7)	2 mo. 8 da.
D_2	0.6 mgm. denatured hormone with 0.3 mgm. Cu.	0.6 mgm. intact hormone with 0.3 mgm. Cu	12 ± 1.2 (7)	3 mo. 8 da.
G	—	0.6 mgm. intact hormone with 0.3 mgm. Cu	23.5 ± 2.4 (8)	3 mo. 15 da.

effect was retained on urea denaturation, some loss may have occurred. A dosage gradient response curve of the antihormone effect is given in figure 2. It was found for groups of mice, as it was for groups of rats, that the maximum ovarian response deviated by 10 per cent from group to group. This was to be expected as 10 per cent corresponds to one standard error. The assay curve given in figure 2 is, therefore, calculated on the percentage decrease of ovarian weight compared with that of control litter mates which received the gonadotropin only at three months. The experiment with the urea-denatured hormone was repeated in two series of experiments. The results showed that 73 ± 22 , 55 ± 12 and 60 ± 20 per cent antihormone activity survived. The standard deviation of these figures are $\sqrt{d_1^2 + d_2^2}$ in which d_1 is the

standard deviation of the mean for the litter mates showing the maximum gonadotropic effect and d_2 is the standard deviation of the mean for the litter mates showing the antihormone effect. In spite of the large standard errors, the three assays of the denatured hormone, compared with intact hormone run simultaneously, indicate the probability in each case of the antihormone activity being greater than 30 per cent in 43 of 44 trials. The smoothness of the reference curve and the agreement between the three unknown assays indicate that the means are influenced by a compensating factor. In view of the fact that the mice are adult and subject to variation in estrus cycle at dosage, variation in response due to this factor might be expected. Since the stage of the cycle would be subject to random sampling the effect would be compensating.

DISCUSSION

The results for the diverse assay procedures of the sheep gonadotropin denatured as a one per cent solution in 40 per cent urea concentration for eight hours at 37.5°C . are as follows: *a) Ovarian hypertrophy*: administered in amounts and under conditions which for the intact hormone produce 800 per cent ovarian hypertrophy in immature rats and 150 per cent ovarian hypertrophy in adult mice, the denatured hormone produced no ovarian hypertrophy; *b) Uterine hypertrophy*: bioassayed in immature rats, the denatured hormone retained 8 ± 1.5 per cent of the original activity; *c) Antagonism phenomenon*: bioassayed by the inhibition which an intraperitoneal injection of hormone exerts to the gonadotropic effect (800 per cent ovarian weight increase in immature rats) of the simultaneous administration of hormone by the subcutaneous route, the denatured hormone retained 11 ± 1.5 per cent of the original activity; *d) Antihormone phenomenon*: bioassayed by the inhibition which a series of injections of hormone administered at two months of age (in Marsh-Buffalo mice) exerts to the gonadotropic effect of the same hormone administered a month later, the denatured hormone retained 63 ± 13 per cent of the original activity.

These results show quite conclusively that the denatured hormone has lost approximately 90 per cent of its gonadotropic and antagonism effects, but retained approximately 60 per cent of its antihormone effect. These results are now considered in light of the dual hormone theory. According to Fevold (12), the interstitial cell-stimulating hormone is as active when given intraperitoneally as when given subcutaneously. Since our assay for antagonist uses the intraperitoneal route and since we have shown that a highly purified follicle-stimulating extract shows the antagonism phenomenon (8), our results in terms of the dual hormone concept would indicate that the denatured hormone does not retain interstitial cell-stimulating properties at the expense of follicle-stimulating properties. If the interstitial cell-stimulating hormone had survived there should have been augmentation. The survival of the antihormone effect could, therefore, not be explained on the basis of survival of interstitial cell-stimulating hormone. We are unaware of any experimental evidence that the interstitial cell-stimulating preparation has been shown to cause antihormone action toward the parent hormone. Our conditions of antihormone assay have been successfully applied to the mare serum gonadotropin (13) which, as before mentioned, retains its multiple properties as a homogeneous protein. The explanation of our results is that in denaturation the gonadotropic effect (and

antagonism phenomenon which is dependent on the gonadotropin powers) is largely lost, but the ability to cause formation of an effective antihormone has been retained.

It has been shown in this laboratory that the antagonism phenomenon is due solely to the rapid liberation of hormone (11), and that the rapid liberation is not conducive to gonadotropic stimulation. Apparently a sudden large onslaught of hormone is overpowering to the recipient cell, curtailing its ability to respond to stimulation. The present studies with the intact hormone add an important observation, that while a plethora of hormone leads to antagonism, it does not materially influence the formation of antihormone. This would indicate that when such a plethora exists destruction of hormone by the organism is not rapid enough to prevent antihormone formation.

The present studies with the denatured hormone also add an important observation in regard to the antihormone phenomenon. Since the denatured hormone under the conditions of administration had no gonadotropic effect, the antihormone effect could not be attributed to a 'burnt out' ovary, that is, one that had been previously stimulated to its maximum response.

SUMMARY

1. Unfractionated sheep pituitary gonadotropin, when subjected to aqueous 40 per cent urea at 37.5° C., rapidly loses its gonadotropic properties so that at six hours' treatment only eight per cent of the original activity survives. Further treatment in 40 per cent urea leads to a gradual loss of potency.

2. Sheep pituitary gonadotropin exposed to aqueous 40 per cent urea for eight hours at 37.5° C. was subjected to three bioassay procedures, viz., the gonadotropic, antagonism and antihormone effects; 8 ± 1.5 of the gonadotropic, 11 ± 1.5 of the antagonism and 63 ± 13 per cent of the antihormone effects were manifested by the denatured product.

3. It is concluded that the multiple physiologic properties manifested by the sheep pituitary gonadotropic fraction are inherent in a single hormone, but that the specific properties are dependent upon the spacing of specific adsorption foci (secondary valence effects), which may or may not be displaced in denaturation. In the example cited, the gonadotropic and antagonist properties which are dependent upon the same foci are largely displaced, but the arrangement required for antihormone formation is largely retained.

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SOME FACTORS INVOLVED IN FOOD AND WATER INGESTION IN THE DOG

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THE physiological problems forming a background for this study are those of urges to ingest food and drink water. The following experiment afforded an opportunity to study several aspects of physiological adjustment of dogs to food and water intakes when a dog chow (Purina), varying in physical form and water content, was administered. The animals were fed the food in block (commercial) form and in pulverized form with and without additional water. The dry food ingested, water drunk and total water ingested were obtained under these different conditions.

Kleitman (1), Bing and Mendel (2), Gregersen (3) and Adolph (4) have reported on some factors involved in water drinking of animals. These studies referred mainly to daily variation in water drunk, relationship of water drunk to food ingested, time of drinking and correlation between body's water deficit and water drunk. Recently Adolph (5) reported results of studies of urges to eat and drink in rats.

METHODS

Four adult female dogs were confined separately to metal cages so constructed that the animals could move about freely. Stationary racks at the sides of the cages supported vessels of drinking water and food. The dogs were acclimated to these cages for periods extending from two to eight months. The only solid food the animals received throughout the entire experiment was dog chow. The water content was determined as 8 per cent (7.98%).

The experiment was divided into three 15-day periods corresponding to the nature of the food administered. These were as follows: *a*) the chow in its usual block form with 8 per cent water, *b*) the pulverized chow with water added so that the total water content was 54 per cent and *c*) the pulverized chow with 8 per cent water. The first period was actually a control period for each dog, but, in addition, *dog 4* was maintained on the block form of the diet throughout all periods as an additional control.

The dogs were fed once daily at a regular time during the first and second periods. They were allowed to eat as much as they desired within a 30-minute interval. The procedure was varied during the third period when the animals were allowed access to the food over a 24-hour interval. This was done since only a very small quantity of the drier pulverized food was eaten within a 30-minute interval, and it was desired that the animals eat a greater quantity of this form of food.

Received for publication February 16, 1948.

The quantity of water in the ingested food was calculated. Water of oxidation was ascertained from the known percentages of carbohydrate, protein and fat in the chow, assuming that these foodstuffs were completely utilized. It seems this assumption is reasonably warranted since there was no appreciable change in weights of the animals over the entire experiment. The volume of water drunk daily by each dog was measured.

RESULTS AND DISCUSSION

Temperature and humidity. The average room temperatures of the first and third 15-day periods were identical, 23.5°C., while that of the second period was 19.0°C. The humidity ranged from 37 to 50 during the entire experiment.

Dry food ingestion. The data for this and subsequent discussion are tabulated in average daily values in table 1. The food intakes, calculated as dry food, of all dogs increased during the second and third periods. The increases were of a large

TABLE 1. FOOD AND WATER INTAKES

	DAILY AVERAGES OF 3 15-DAY PERIODS, ¹ IN GRAMS											
	1	2	3	1	2	3	1	2	3	1	2	3
	<i>Dog 1</i>			<i>Dog 2</i>			<i>Dog 3</i>			<i>Dog 4, control²</i>		
Dry food ingested	82.1	122.4	97.7	90.3	142.2	103.6	91.7	144.8	122.3	82.9	128.4	102.4
Water in food ingested	7.1	143.7	8.5	7.9	166.9	9.0	8.0	169.9	10.6	7.2	11.2	8.9
Calculated water of oxidation	35.3	52.7	42.0	38.9	61.2	44.6	39.5	62.3	52.6	35.7	55.2	44.0
Water drunk	194.7	151.6	228.0	148.9	202.9	192.6	247.8	273.5	313.2	204.6	302.3	300.3
Total water ingested	237.1	348.0	278.5	195.7	431.0	246.2	295.3	505.7	376.4	247.5	368.7	353.2

¹ Period 1. Block chow containing 8% water; Period 2. Pulverized chow containing 54% water; Period 3. Pulverized chow containing 8% water.

² Control dog received Block chow containing 8% water during all three periods.

order, varying from 49 to 58 per cent in the second period and 19 to 33 per cent in the third. The magnitude of the increase in the second period was due probably to the lowered room temperature, since also the control dog ate more food proportionately during this interval. More dry food was ingested accompanying increased body metabolism.

It is interesting that the dry food intakes of the three experimental animals during the third period were 19 to 33 per cent greater than those of the first period. In the first period the animals had 30 minutes' access to the food, whereas in the third the interval of access was 24 hours. Questions may be raised as to why the drier pulverized food was not ingested as quickly as when the food was in the block form or contained more water. Does pulverization of the drier food make a greater demand on salivary secretion, and to what extent are rate and quantity of food ingestion dependent on salivation? If the drier pulverized food makes a greater demand on salivation and this is a factor in the rate of ingestion it would seem the dog meets this situation well if it has a sufficient interval of access to the food.

Total water ingestion. The total water ingestion of all dogs (including control)

increased during the second and third periods as contrasted with the first. The range was from 46.8 to 120.2 per cent in the second period and from 17.5 to 42.7 per cent in the third. The greatest increase in total water ingestion occurred when the food contained a greater percentage of water. In general, increased ingestion of dry food was accompanied in both periods by increased total water consumption, but there was no correlation between the ratio of total water intake to dry food ingested. The data indicate there is a wide variation in the total amounts of water consumed by different dogs. Evidently there is no fixed relationship between total water drunk and dry food ingested, and the animal does not seem to limit itself rigidly to total water intake.

RATIO OF WATER DRUNK TO DRY FOOD DIGESTED

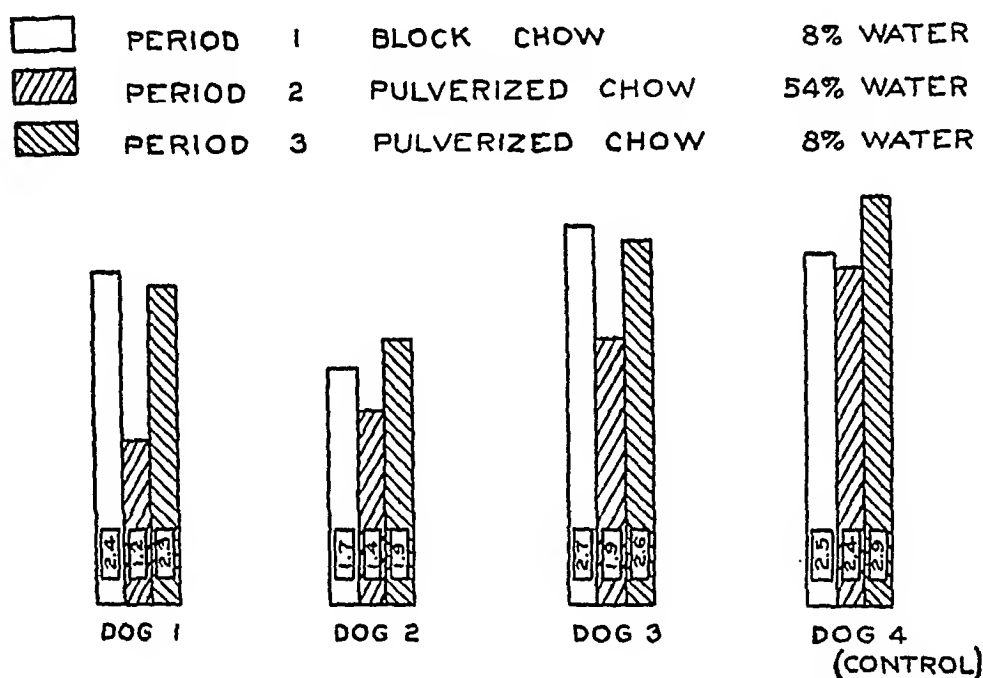


Fig. 1. COMPARISON OF WATER DRUNK to dry food ingested in all periods

Water drunk. The ratio of water drunk to dry food ingested in the second period decreased markedly in two dogs, as shown in figure 1. This suggests that the dog may meet part of its water requirements from water in the food rather than from water drunk when the food contains sufficient water.

A striking feature of this phase of the experiment is that the ratio of water drunk to dry food ingested is practically the same in the third as in the first period. Pulverization of the food did not affect appreciably the volume of water drunk. If ingestion of the pulverized food resulted in increased salivary flow apparently there was no accompanying urge to drink.

Dog 3 ingested greater quantities of food and water than any of the other dogs during the entire experiment. The increased food and water consumption in this

animal may have been due to the growth of a mammary tumor which began to appear when the study was about one third completed. This tumor developed extremely rapidly during the remainder of the experiment.

SUMMARY

Dogs were placed on a chow diet which was administered in block form and in pulverized form with and without additional water. The amounts of dry food ingested, water drunk and total water consumed were determined. It was found that increasing the water content of the comparatively dry pulverized food facilitated ingestion, yet ingestion of the food without addition of water did not affect appreciably the urge to drink, as evidenced by the amount of water drunk. In addition, water requirements were met, in part, at the expense of drinking water, and finally, the total water intake of the animal was not uniform nor related directly to any one known factor.

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B VITAMIN REQUIREMENTS WITH ADVANCING AGE

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TWO years ago (1) we reported a markedly increased thiamine requirement (in mgm. per kgm. of diet) with advancing age in rats. Since then, Rafsky and Newman (2) have described an apparent thiamine deficiency in aged people on diets which are qualitatively quite adequate for young adults. Low blood thiamine levels and low urinary output in elderly people on what was formerly considered an adequate thiamine intake led these authors to conclude that diets for the aged need enrichment.

During the last two years we have followed a group of rats from weaning time to old age, periodically testing them for their requirement of one B vitamin after another needed for optimal growth. In this way we hoped to discover any other changes in requirement similar to that for thiamine. No changes were found except for verification of the marked rise of thiamine requirement in old age. Dietary concentrations of the other B vitamins adequate for optimal growth in young animals remain similarly adequate throughout life.

Eighty Sprague-Dawley rats (males) were acquired at three weeks of age and divided evenly between tropical moist warmth (90-91°F. and 60-70% relative humidity) and temperate coolness (68-70°F.); for testing, the 40 rats in each room were divided into four groups of 10 each. When not on test diets, they were fed exclusively on Purina dog chow (large checkers). When reassembled into new groups for the next test, care was taken to obtain the most even possible division of the rats from each of the preceding four groups among the four new groups being formed, thus minimizing the disturbing influence of previous nutritional differences.

The basal diet used throughout the study consisted of: sucrose 76%, vitamin-free casein 18%, corn oil 2% and salt mixture (3) 4%. Vitamins added (per kilo of diet mixture) were: haliver oil 1.2 cc., thiamine chloride 2 mgm. in cold and 3 mgm. in heat, riboflavin 4 mgm., pyridoxine 4 mgm., calcium pantothenate 6 mgm., nicotinamide 25 mgm., p-aminobenzoic acid 0.3 gram, choline chloride 0.75 gram, a-tocopherol 0.5 mgm. vitamin K 0.5 mgm. folic acid 1 mgm., and inositol 1 gram. In testing for optimal requirements for a given B vitamin, the above amount for that particular one was replaced by the graded amounts as indicated in table 1.

From the results set forth in table 1, it is evident that advancing age brings no significant change in rat requirement for pyridoxine or riboflavin. Weight gain at 1.0 mgm. of pyridoxine per kilo of diet was practically as good as at any higher level at a rat age of four months and at the advanced age of 18½ months. The same held for riboflavin at rat ages of 6 and 13½ months. These requirement levels check very well with those previously reported by us for weaning rats (4) with no significant difference between the rats kept in tropical moist warmth and in temperate coolness.

Received for publication January 5, 1948.

TABLE 1.—Continued

Age at completion of test	Room conditions:		68-70°F.				90-91°F. and 60-70% relative humidity			
	Dietary pyridoxine (mgm./kgm. of diet)		0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
18½ months	Pyridoxine (mgm./kgm. of diet)									
	Wt. gain (g.) last 3 of 5 wks. on diets		-24.27 ± 16.69	+15.00 ± 19.61	+18.33 ± 25.16	+20.00 ± 25.16	-12.80 ± 13.34	+21.25 ± 13.48	+11.50 ± 10.83	+10.00 ± 17.66
	Final weights (g.)		407.87 ± 12.03	510.00 ± 12.16	555.00 ± 14.47	550.00 ± 19.15	485.00 ± 10.01	481.25 ± 10.43	502.50 ± 8.04	555.00 ± 12.49
	Food intake (g./rat/wk.)		123 (7)	122 (6)	141 (6)	153 (6)	79 (7)	78 (8)	91 (8)	103 (7)
24½ months	Thiamine (mgm./kgm. of diet)									
	Wt. gain (g.) last 4½ wks. of 7½ wks. on diets		-9.00 ± 46.66	-18.00 ± 23.99	0.0 ± 18.26	1.0	1.5	2.0	2.5	3.0
	Final weights (g.)		535.00 ± 34.40	460.00 ± 14.87	550.00 ± 14.57	550.00 ± 14.57	535.00 ± 34.40	2 of 4 rats died on the 8th day of diet.	-30.00 ± 24.32	+5.00 ± 11.35
	Food intake (g./rat/wk.)		132 (5) ¹	112 (5)	136 (4)	136 (4)	132 (5)		485.00 ± 20.23	565.00 ± 8.26
									113 (3)	88 (4)

¹ Numbers of rats per group are indicated in parentheses.

The sharp increase in choline requirement previously reported for weanling rats—to prevent hemorrhagic nephritis and maintain optimal growth (5)—does not seem to be present at more advanced ages in either heat or cold. At $8\frac{1}{2}$ months of age the rats seemed insensitive to pantothenic acid variations from 8 mgm. down to 2 mgm. per kilo of diet.

Tested at $24\frac{1}{2}$ months of age, when over half of the original animals had died of old age, this second rat series gave indication of the same increase in thiamine requirement previously reported (1). For optimal response in old age, rats seem to require about two and one half times as much thiamine per kilo of food mixture as is needed for best growth of weanlings. In this series, as in the previously reported one, death in acute thiamine deficiency occurred in the $24\frac{1}{2}$ month-old, hot room rats placed on diets containing 2.0 mgm. of thiamine per kilo of diet, a thiamine level which is more than adequate to support optimal growth in weanlings (6).

Six of the cold room rats developed tumors (unidentified) between the ages of 16 and 18 months, at a time when only 25 were still surviving out of the original 40. Only one tumor developed in the hot room group, when the 15 remaining survivors were 22 months old.

SUMMARY

The increase in thiamine requirement (in mgm. per kgm. of food mixture) previously reported with advancing age in rats seems to receive further verification. None of the other B vitamins studied (choline, pyridoxine, riboflavin and pantothenic acid) gave any evidence of similar increase in requirement with advancing age. These findings, in conjunction with the studies on aged people (2), provide ample reason to recommend thiamin supplementation for elderly people. There is no basis yet for similarly supplementing the normal dietary intake of other members of the B group.

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RAT COLONY TESTING BY ZUCKER'S WEIGHT-AGE RELATION¹

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A VISUAL test of the 'normality' of a rat colony, applicable without appreciable expenditure of time and requiring almost no numerical calculation, is given by the observation of Zucker and his collaborators (1) that if the body weights of rats are plotted on semi-log paper against the reciprocals of their ages, the points should fall along a straight line. This is shown to be true for three colonies of rats by Zucker, and for nine colonies in figure 1 of this paper.

By contrast, the phenomenon is not true of Donaldson's colony (2), the very one that has been most carefully studied and that has most often been referred to as a standard. This discrepancy from later colonies was shown by Zucker in his figure 2, where the line is convex downward between about 28 and 105 days of age and only later becomes a straight line. When body weight is plotted against age, Donaldson's measurements give that sigmoid curve, with a point of inflection which seems to lie at about 66 days of age, that used to be regarded as characteristic of postweaning growth in the rat. The plots of all later colonies, however, show a body weight that rises in a smooth unbroken curve through all ages. Since Donaldson worked at a time when there was less knowledge than we now possess as to what constitutes a complete food for the rat, it may be that his sigmoid curve, which manifests the deviation of his data from the straight line relation between the log of the body weight and the reciprocal of age, is an indication that his diet, though adequate for adult rats, was not optimal for young rats and was more and more inadequate the younger the rat and the greater the rate of growth.

If it can be shown that unimpeded growth in the rat always follows Zucker's easily tested rule, the log-reciprocal relation should be a useful tool for every one who intends to use body-weight change as a measure of the effect of any experimental variable, useful before, not after, the measurements are made, and useful in the same manner that an inquiry into the purity of his reagents is useful to the chemist. For it is not true, as has unfortunately often been supposed, that all other variables than the one to be tested are excluded by setting aside an adequate number of rats as controls. There is an interaction between variables (3), and a colony under the influence of some unsuspected variable, a dietary deficiency or a pathological process may react very differently relatively to its controls than another colony that is free from such anomalies.

The present paper gives the result of a study of this relation in females in nine colonies from sundry laboratories (fig. 1), and in males in three samples in our own colony (fig. 2). The desired comparison is easily made visually without calculation. And for those who need details, the conventional parameters (means and variabilities) are recorded in tables 1 and 2 and discussed in the text.

The slope k , both for ordinary albinos and Mendel's big-bred albinos, was rea-

Received for publication March 24, 1948.

¹ This work was aided by a grant from the Nutrition Foundation, Inc.

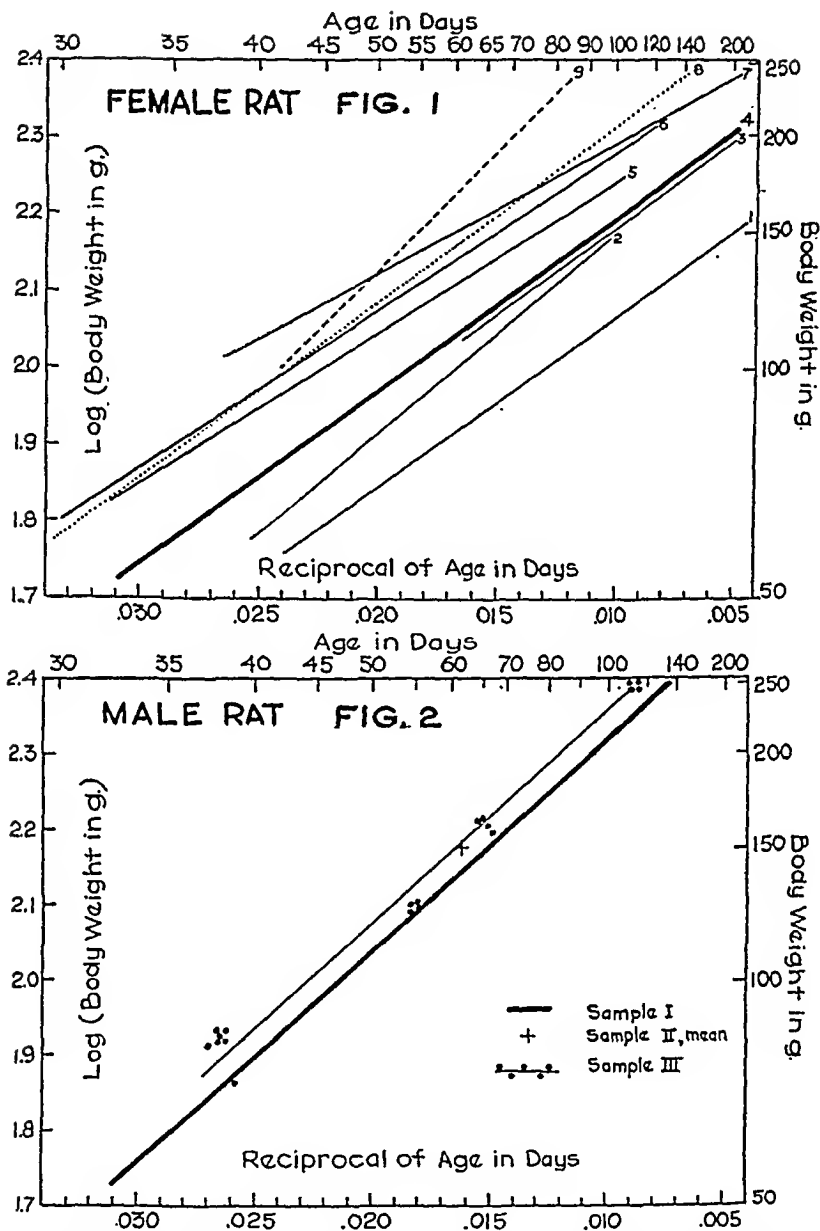


Fig. 1. COMPARISON BETWEEN COLONIES. Albinos: 1 Jackson; 2 Hanson and Heys; 3 King; 4 Gray and Addis' Sample I; 5 Zucker; 6 Maynard; 7 Smith and Bing; 8 *Evans-Long* hybrid rats untreated, raised by Addis; 9 *Evans-Long, pituitary-treated* reported by Zucker, and here dashed to emphasize that they were different from the others qualitatively. Albinos bred for size by Mendel and Hubbell are not shown, having been discussed by Zucker. Sperry gave no data for females and Macy's line was omitted in the graph; hence though table 1 has 11 lines, figure 1 has only 9.

Fig. 2. COMPARISON OF THREE SAMPLES WITHIN ONE COLONY. Males only. Sample I: 291 earlier albino males, that is, through 1940, the heavy line. Sample II: 1600 albino males from experiments in Laboratory Book 108, which will be reported elsewhere; since animals were selected by bodyweight, all 150 grams (more exactly 148 to 152), no regression line on age was feasible, but the average age, 61.8 days, was used and the whole sample mean therefore plotted at one central point X, with the conclusion that in this sample body-weight for a given age was heavier than in sample I. Sample III: 24 recent albino males, a dot for each and the thin line for the equation fitted to them by least squares; conclusion that the sample, like II, had with time developed heavier weight for a given age than sample I. This secular change is noteworthy.

sonably constant for males but not so uniform for females. Just why the coefficient of variability is twice as big in the latter we cannot say. In both sexes, however,

TABLE 1. CONSTANTS OF LOG-RECIPROCAL EQUATION

Colony	Date	Males				Females				Sex Ratio for k, M/F	Sex Ratio for A, M/F
		N	Age range in days	k	A	N	Age range in days	k	A		
Jackson	1913	67	42-365	28.02	265	84	42-365	22.50	198	1.25	1.34
King	1915	554	60-485	27.75	367	537	60-485	21.78	255	1.27	1.44
Hanson & Heys	1927	1034	40-100	31.69	396	1169	40-100	26.52	279	1.19	1.42
Macy	1927	n.s.	28-210	25.62	484	n.s.	28-210	20.02	278	1.28	1.74
Smith & Bing	1928	1260	30-239	26.74	573	506	35-239	16.81	296	1.59	1.94
Maynard	1930	n.s.	28-196	26.81	535	n.s.	28-196	20.51	314	1.31	1.70
Freudenberger	1932	n.s.	21-448	25.90	440	n.s.	21-448	19.60	282	1.32	1.56
Sperry-Stoyanoff III	1934	n.s.	21-154	25.76	476						
Mendel & Hubbell	1935	n.s.	21-273	26.11	650	n.s.	21-112	19.88	380	1.31	1.71
Zucker <i>et al.</i>	1941	42	28-119	25.55	470	311	28-200	19.88	270	1.29	1.74
Addis	1948	921	32-260	27.92	398	925	32-260	21.97	260	1.27	1.53

$$\log_{10}(\text{body-weight in grams}) = -k \frac{1}{d} + \log A,$$

where k = slope of the straight line, 1/d = reciprocal of age in days, A = estimated asymptote or limit approached by body-weight, n.s. = not stated.

TABLE 2. MEANS AND VARIABILITIES FOR THE LINE CONSTANTS

	Sex	N	Mean	Standard De- viation, SD	Standard Error of Mean, SE	Coefficient of Variation, CV
Slope, k	Male	10	27.18	1.86	.587	6.8
	Female	9	21.07	2.63	.877	12.5
Asymptote, A	Male	10	440	88	28	20.1
	Female	9	270	32	11	12.0
Sex ratio for k	M/F	9	1.31	.112	.038	8.6
Sex ratio for A	M/F	9	1.60	.193	.064	12.0

These constants were calculated from the various items in table 1, omitting Mendel's sample bred for bigness. Formulae used in computing: the standard deviation SD was calculated by Fisher's formula which uses, not the number of cases N, but the degrees of freedom df, which here is

$$N-1; \text{ hence } SD = \sqrt{\frac{S(x^2) - (Sx)^2/N}{df}}.$$
 The standard error of the mean, $SE = SD/\sqrt{N}$.
The coefficient of variation, $CV = 100 \text{ } SD/\text{Mean}$.

one may say that there is a family of lines with the slope of approximately 27 in males and 21 in females. Viewed this way, the slope is less interesting for the investigator to watch than the intercept which is log A. If that intercept is larger, one would have to say that there had been a significant change in the sample as compared with

another sample, and then one would have to decide from other evidence whether the cause was secular or dietary or some other factor in the investigation.

The coefficient of variability of A is so large that it is evidently unsafe to think of this asymptote as merely about 440 grams for males and 270 grams for females; but one should select the sample nearest one's own as a base of first comparison.

The original data from which Zucker and his associates derived their relation were published in a table showing a rather small sample of 42 male and 311 female albinos, of ages from 28 days (4 weeks) to 119 days (17 weeks). Also there were given the important constants or parameters of body-weight for age (his class intervals being one week), namely the mean and the standard deviation in grams, and the coefficient of variability in percentage of the mean. The last named constant is too often ignored, with the unfortunate result that an observation, or even a mean of observations, appears on a diagram to indicate a material trend, when in reality it should be discounted as representing merely a fluctuation due to the universal variability of biological matter. However, Zucker did not fail to note the wide fluctuation, which in some age groups was only 8 per cent and in others as much as 18 per cent; also these variabilities characterized, not their colony as a whole, but only those particular data on breeding stock animals selected at weaning so that the weights clustered around the chosen means. This reservation unfortunately restricts the usefulness of their values for purposes of comparison.

Formulation was their next consideration. They remarked, and we agree, that growth curves have a point of inflection, but it comes before weaning, which in our colony occurs at 25 days of age. Instead of the strongly marked sigmoid form of older data, the simple form with constant slope warranted transformation into a straight-line function for greater ease of interpretation and comparison. Such an empirical transformation, if it leads to a simple equation, is always useful but need not have any reference to theoretical interpretation of the growth process. Their transformation, then, was this: If the logarithm of weight is plotted against the reciprocal of time, a good straight line results. Their formula for this straight line was: $\log W = -k/t + \log A$, in more familiar notation $Y = B + kX$, where $X = 1/t =$ the reciprocal of time or age, W is the weight in grams, $Y = \log_{10} W$, A is the weight approached asymptotically in the adult ($\log A = B =$ the intercept of the straight line), and k is the slope of the line which characterizes the rate of growth, $k = (\log W_2 - \log W_1)/(1/t_1 - 1/t_2)$. The theoretical underlying principles and some applications were discussed by Zucker and later by Dunn (4).

The asymptote A is the body-weight approached during growth. It is a statistical limit rather than a value one expects to find. Furthermore it can be the same for two samples which have different slopes; e.g., in table 1, the females of Hanson and Heys have essentially the same A as those of Macy, but the slope k for the former colony is 28 per cent larger than for the latter. Macy's colony is not graphed in figure 2 because this discrepancy in slope would confuse visualization of the principal phenomena.

One may be interested in the size of A as such. Primarily one is concerned with obtaining a line to put on a graph, because the individual animals observed and plotted are to be examined, not in relation to the theoretical limit of the oldest, heaviest animals but in relation to the line, i.e., to animals of like age.

RESULTS

In table 1 we give the constants of the log-reciprocal equation derived from 11 colonies. These were computed from published data for albino rats, using days rather than weeks of age as the unit of time. In figure 1 lines are given for females in seven albino colonies selected to show the great differences in the size of the animals at any given age. For all colonies that we have examined, except Donaldson's, straight lines are obtained for the relation and we can thus confirm Zucker's rule. Figure 1 shows the degree to which the slopes vary. In all cases except one the lines represent supposedly normal growth. In this exception, line 9 of figure 1, an experimental variable, the injection of growth hormone had been introduced, and here it can be seen that though the line is still straight its slope is greatly changed. The parallelism between line 8 (Evans-Long) and line 4 (our albinos), in spite of the considerable difference in body size, is presumably a consequence of the fact that both of these colonies were born and grew up on the same diet and under the same complex of external conditions. The fathers and mothers of these Evans-Long rats came from Dr. Herbert Evans' colony in Berkeley, and their children were examined in order to determine whether or not this relation, and other relations between body-weight and organ-weight that characterize our colony, were affected by the absolute body-size difference. The parallelism between line 4 and line 8 in figure 1 shows that k remains constant in spite of a marked difference in A . Within albino colonies differences in A are presumably a matter of conscious or unconscious selection for size. When an explicit attempt is made to breed for size, as in the case of Mendel and Hubbell's colony, table 1 shows that A may rise to 650 grams for males and 380 grams for females as compared with an average-sized colony such as ours where the respective A constants are 398 and 260 grams.

When there is no intentional selection for size but simply the rejection at the time of weaning of small ill-nourished animals because it is thought they may be diseased or anomalous, there is, over the years, a gradual increase in the absolute size of rats in any one colony. This is shown for males of our colony in figure 2. Sample I shows the line calculated from the values already given for 921 rats in table 1, covering the period 1929 to 1940. Sample II covers the period 1945 to 47; it represents 1600 animals, which for the purposes of the experiments then in progress were selected so that their weights were close to 150 grams, hence no regression line was possible. The mean body-weight was 150.3 grams and the mean of the ages was 61.8 days, with the large range of 52 to 82 days and standard deviation of 5 days; these two means are therefore plotted as a cross. Sample III was composed of 24 rats taken from the current colony at random; their weight ranged from 68 to 286 grams, and a line fitted yielded the parameter $k = 28.21$, asymptote $A = 439$ grams, vertical intercept or $\log A = 2.642$.

Comparison of these three samples within the same colony is simple visually in the graph, or by taking a convenient age, say 61.8 days, and solving the equations for I and II. Then the corresponding body weights for the three samples are 140.6, 150.3 and 153.5 grams. Easy comparison of these can then be made by taking I as a base, with sample II 10.7 per cent heavier and III 10.9 per cent heavier at the stated age. Hence the latter two samples may be considered identical, but notably heavier than the earliest sample.

Practical procedure. It is to be emphasized to the investigator that the relationship is to be considered in two stages: one the simple visual method for use during an experiment, the second the later defining of a particular sample by calculations for purposes of record.

The simple visual method requires only ordinary cross-section paper, on which the horizontal or X scale is laid off in working units of reciprocals. For example, if the age range of one's sample runs from 30 to 200 days, the corresponding reciprocals from .033 to .005 are inscribed, beginning at the left. Then the vertical or Y scale of the body-weights in the sample range from 50 to 250 grams, and the appropriate logarithms from 1.7 to 2.4 are laid off. This arrangement is seen in our two diagrams, figures 1 and 2. For those who prefer avoiding a table of logs but do not mind the finer gradations, semilog paper with two decks on the vertical axis can of course be used.

The experimenter then plots the observations as they are made and soon will see whether the expected straight line can indeed be fitted by eye. We wish then to insist on this simplicity.

Later, if desired, the investigator can solve, at three ages, the equation reported here for any colony which seems to him comparable with his own, and from those three points draw in the line on his plot. And soon he will have such a line of his own as a standard for his future experiments.

SUMMARY

A straight line relation between the log body-weight and the reciprocal of age has been found to hold for data derived from 11 different colonies, and the constants of the equation have been determined. The slope of the line varies moderately in different colonies. Since it is markedly altered when growth rate is changed by growth hormones we suppose that the differences in slope between the different colonies may be derived from the action of individual factors that have the same relative effect at all ages. The deviation from a straight line in Donaldson's data may be a consequence of a factor acting only at certain ages, as for instance a diet adequate for old but not optimal for young rats.

The A constant in the equation, the maximum size approached asymptotically by a colony, varies widely. This may be a consequence of hybridization of two strains of different sizes, as in the Evans-Long colony, or may be a result of selection for size as in the Mendel-Hubbell colony. It is shown that in spite of wide differences in A , k remains constant when the environmental conditions are the same. Secular changes in the A constant are shown to occur in a colony in which there was no intentional selection for size, presumably as a consequence of the rejection of unusually small or ill-nourished rats at the time of weaning.

The log-reciprocal relation is an easily applied index to test the operation on a colony of unsuspected variables and may be used as a measure of secular change in a colony.

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EFFECT OF ORALLY ADMINISTERED UREA ON THE AMMONIA AND UREA CONCENTRATION IN THE BLOOD OF CATTLE AND SHEEP, WITH OBSERVATIONS ON BLOOD AMMONIA LEVELS ASSOCIATED WITH SYMPTOMS OF ALKALOSIS¹

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DURING recent years considerable interest has been shown in the possible use of urea to supply part of the nitrogen (protein) needs of cattle and sheep (1, 3, 4, 5). Evidence has accumulated to show that ruminants are able to utilize some urea nitrogen for productive purposes, although the exact mechanism of this utilization is not known. It has been suggested (6) that bacteria in the rumen are responsible for conversion of the urea nitrogen to a form which can be utilized by the host. If such a conversion takes place, the rate of absorption of urea should be a factor affecting the efficiency of its utilization, since that which is absorbed is no longer subject to bacterial action. Studies have been made at this experiment station with sheep and cattle to determine the rate of absorption and possible toxicity of urea when administered orally in water solution and when mixed with concentrate feeds in a practical ration. Urea and ammonia were determined in both the portal and systemic blood of a sheep in one experiment and in the systemic blood of steers in all other experiments.

EXPERIMENTAL

The sheep used for the portal blood studies was a ram lamb weighing 90 pounds which had been on full feed of hay and grain. Twenty-four hours before the experiment all roughage was taken from the ration in order to reduce the rumen and intestinal contents. The lamb was anesthetized with pentothal sodium injected intravenously, with supplements of ether given during the experiment. An incision was made in the right abdominal wall just posterior to the costal margin and the portal vein exposed by retraction of the abdominal viscera. Samples of portal and systemic blood were drawn immediately. Forty grams of urea and 40 grams of sucrose dissolved in 500 ml. of water were then directed into the rumen by means of a stomach tube. Samples of portal blood were taken at 15-minute intervals thereafter for two hours and a sample of systemic blood from the jugular vein was taken at the time of the last portal sample. Urea and ammonia were determined by the method of Van Slyke and

Received for publication February 2, 1948.

¹ This study was in part financed through a research grant from the E. I. du Pont de Nemours and Co., Inc., Wilmington 98, Delaware.

Cullen (7), all determinations being completed within four hours after the first sample was drawn. The blood values are given in table 1.

Four yearling steers weighing approximately 500 pounds each were used for the study of ammonia and urea levels in systemic blood following oral administration of water solutions of urea. Prior to the experiment the steers were on full feed of 8 pounds of prairie hay and 3 pounds of cottonseed meal daily. The

TABLE 1. UREA AND $\text{NH}_3\text{-N}$ IN THE PORTAL AND SYSTEMIC BLOOD OF A SHEEP FOLLOWING ADMINISTRATION OF 40 GRAMS OF UREA AND 40 GRAMS OF SUCROSE (values in mgm. N per 100 ml. of blood)

ITEMS COMPARED	TIME IN MINUTES								
	0	15	30	45	60	75	90	105	120
NH ₃ -N in portal blood.....	1.75	4.06	4.75	3.50	6.65	6.51	5.60	7.70	8.40
NH ₃ -N in systemic blood.....	0.56								3.36
Urea-N in portal blood.....	7.77	15.26	13.31	14.07	13.86	13.51	14.56	14.77	15.12
Urea-N in systemic blood....	8.40								16.10

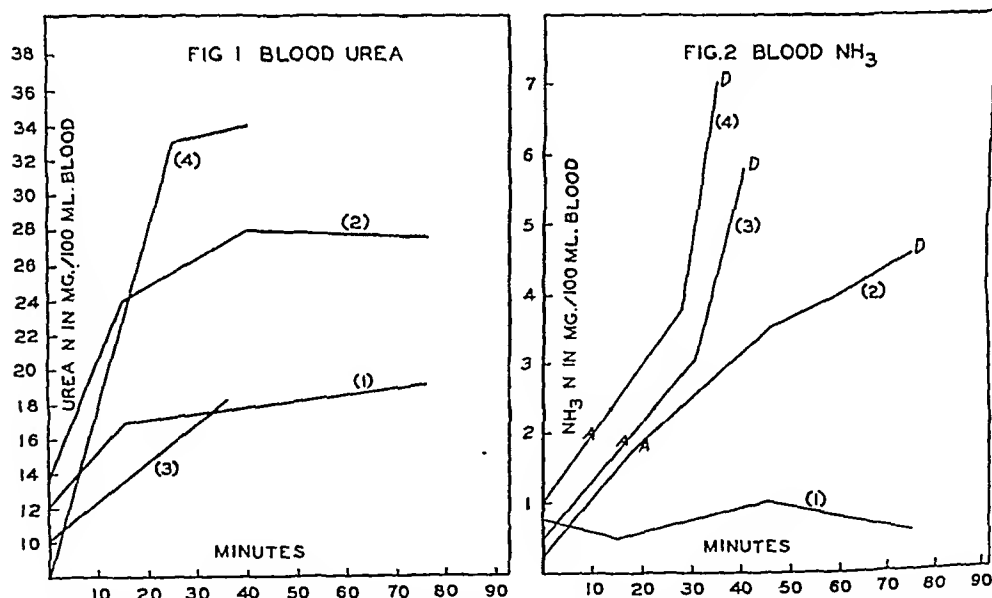


FIG. 1 and 2. EFFECT OF ADMINISTRATION OF WATER SOLUTIONS of urea on blood urea and NH_3 levels in cattle. Steers 1, 2, 3, and 4 received 57, 114, 272, and 490 grams of urea, respectively. A represents the point at which ataxia was first observed; D represents death of the animal.

morning on which urea was to be administered the steers were fed 4 pounds of concentrate containing 50 parts cottonseed meal, 32 parts hominy feed and 10 parts blackstrap molasses. When the animals had consumed their feed, blood samples were obtained by jugular stab to determine the initial blood urea and ammonia values. Urea in varying amounts dissolved in a liter of water was then given orally by means of a short rubber tube 25 cm. in length which reached

to about the midpoint of the esophagus. Steers 1, 2, 3, and 4 received 57, 116, 272 and 490 grams of urea, respectively, in this manner. Urea and ammonia determinations were made on jugular blood samples taken at frequent intervals until death or recovery of the animals. The results are presented graphically in figures 1 and 2.

In the foregoing experiment the blood ammonia values increased rapidly and with fatal results. Therefore, it appeared desirable to continue the study by feeding urea mixed with a concentrate feed. The concentrate feed used was composed of cottonseed meal, hominy feed and molasses in the same proportions as given above plus eight parts of urea. Four steers were fed 40, 48, 73, and 80 grams of urea in this manner and a fifth steer was given the same basal ration

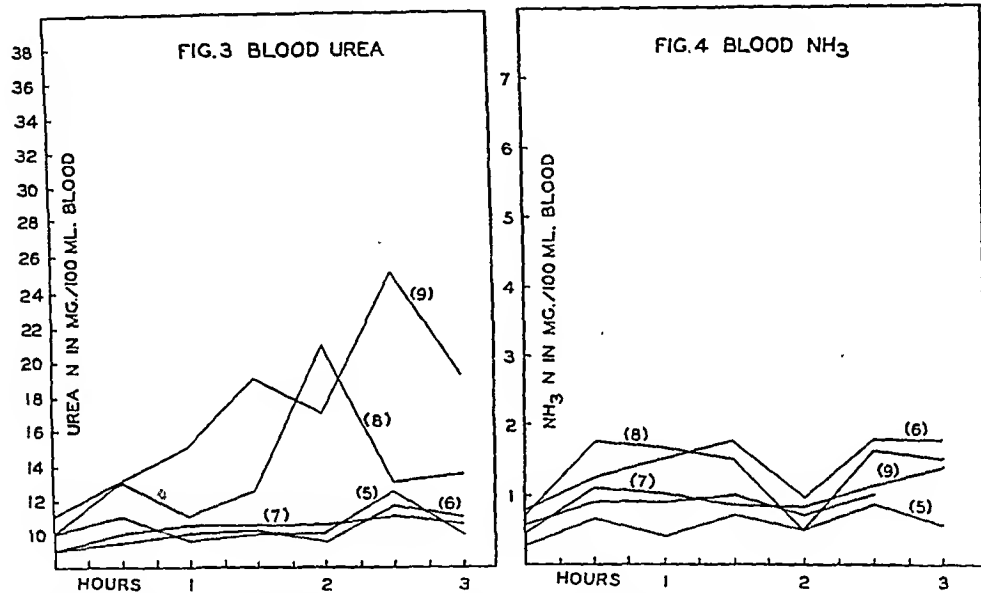


FIG. 3 and 4. EFFECT OF UREA EATEN IN MIXED FEED on blood urea and NH₃ levels in cattle. Steer 5 received the basal ration without urea. Steers 6, 7, 8, and 9 ate 40, 48, 73, and 80 grams of urea, respectively.

without urea. Attempts to induce the steers to eat larger amounts of urea in a short period of time failed. Urea and ammonia determinations were on jugular blood samples taken immediately after the steers had consumed their feed and at 30-minute intervals during the next three hours. The results are given in figures 3 and 4.

Two additional experiments were then carried out. In one of these, the amount of urea fed in a mixed feed was gradually increased. In the other, a high urea ration was suspended in water and given as a drench.

RESULTS AND DISCUSSION

Table 1 presents values for urea and ammonia nitrogen in the portal and systemic blood of the sheep immediately preceding and following oral administration of urea in water solution. Portal urea-N values increased from 7.77 mgm.

per cent to 15.26 mgm. per cent within 15 minutes after the urea was given. During the same period portal $\text{NH}_3\text{-N}$ values increased from 1.75 to 4.06 mgm. Thereafter, the urea values remained fairly constant whereas the $\text{NH}_3\text{-N}$ values continued to increase until at the end of two hours a value of 8.40 mgm. was reached. Changes of similar magnitude were observed in the urea = N and $\text{NH}_3\text{-N}$ value of the systemic blood at the end of two hours. The increase in $\text{NH}_3\text{-N}$ of the portal blood indicates absorption of appreciable quantities of ammonia produced by the hydrolysis of urea in the gastrointestinal tract.

Changes in blood urea and ammonia produced by oral administration of from 57 to 490 grams of urea to steers are shown in figures 1 and 2. When 57 grams of urea were given to *steer 1*, urea-N values increased to about 18 mgm. per cent during the next 70 minutes while $\text{NH}_3\text{-N}$ values varied only slightly from the initial value of one mgm. per cent. When 116, 262, and 490 grams of urea were given to *steers 2, 3, and 4*, respectively, there was a rapid rise in both urea and ammonia and death followed within a period of 70 minutes. At the time of death, $\text{NH}_3\text{-N}$ values of *steers 2, 3, and 4* had reached 4.48, 5.74, and 7.14 mgm. per cent, respectively. Urea-N values, although high, at no time exceeded those which have been observed in other animals consuming high-urea rations without ill effects. The three steers exhibited almost identical symptoms preceding death. Ataxia, especially of the front legs, occurred within 20 minutes after the urea was given. Ammonia-N values at that time had reached about 2 mgm. per cent. The steers became unable to stand and went into severe tetany. Respiration became slow and difficult with frequent gasping, and the animals exhibited excessive salivation with frothing. As blood ammonia values continued to rise the tetany became progressively worse. The highest $\text{NH}_3\text{-N}$ value observed during the periods of survival was 3.74 mgm. per cent for *steer 4* at the end of 24 minutes. The lowest value observed at the time of death was 4.48 mgm. per cent (*steer 2*). It is suggested, therefore, that the lethal level of $\text{NH}_3\text{-N}$ in the blood is somewhat below these levels but above 2 mgm. per cent. Bang (2) reports that for rabbits the lethal level is about 4 mgm. per cent.

In an attempt to alleviate the tetany of a steer which had been given 256 grams of urea by stomach tube, 75 grams of a mixture of equal parts of calcium chloride, magnesium chloride and dextrose were given intravenously. The tetany completely disappeared within 15 minutes after the injection but the steer died one hour and thirty minutes later.

Another steer was given 236 gram of urea in water by means of a stomach tube of such length that it could be palpated in the rumen. This longer tube was used to insure delivery of the urea into the rumen with little opportunity of its being shunted into other stomach compartments. In the following two hours urea-N of systemic blood increased from an initial value of 18.40 mgm. per cent to 28.63 mgm. per cent; $\text{NH}_3\text{-N}$ increased from 1.12 to 1.82 mgm. per cent. The steer showed none of the symptoms described for those given urea with a short tube, but died seven hours later. Autopsy revealed severe inflammation of the wall of the rumen in the region in which the tube was palpated. The absence of symptoms of alkalosis, relatively low $\text{NH}_3\text{-N}$ values, and delayed

death of this animal indicate a difference in the effect of urea when directed into the rumen and when directed into the esophagus from where it might by-pass the rumen.

The effect of urea eaten in feed on the urea and ammonia concentration of the blood of steers is shown in figures 3 and 4. When given in this manner 73 and 80 grams of urea eaten by *steers 8* and *9*, respectively, produced a slow rise in the urea-N blood level which reached its peak of about 24 mgm. per cent in from 2 to 2½ hours. The initial rise in urea-N was less rapid than that observed when comparable amounts of urea were given to *steers 1* and *2* by stomach tube. Smaller amounts of urea consumed by *steers 6* and *7* produced no significant change in blood urea. Ammonia-N levels for all steers remained below 2 mgm. per cent. Although the steers refused to eat urea in as large amounts as had been given some animals by stomach tube it was possible by gradually increasing the percentage of urea in a mixed feed to induce one steer to eat 400 grams of urea daily for 70 days. Approximately five hours were required for the steer to eat the ration. Blood samples taken two hours after the steer had eaten contained 30.10 mgm. per cent of urea-N and 0.70 mgm. per cent of $\text{NH}_3\text{-N}$. At no time did this animal show symptoms of distress as a result of this high urea intake, although similar amounts and even smaller amounts of urea had proved fatal when given by stomach tube. In feed lot experiments eight steer calves have gained weight and shown no symptoms of toxicity on rations which supplied 200 grams of urea daily.

Since dilution of urea with mixed feed appeared to favor its slow hydrolysis and absorption, a suspension of 5 pounds of the basal ration, previously described, plus 180 grams of urea in 4 gallons of water was directed into the rumen of a steer by means of a long stomach tube. Blood urea-N values determined an hour later increased from an initial value of 12.95 mgm. per cent to 21.77 mgm. per cent; $\text{NH}_3\text{-N}$ at that time was 2.52 mgm. per cent. The steer exhibited the characteristic ataxia and tetany of animals with blood $\text{NH}_3\text{-N}$ values above 2 mgm. but appeared perfectly normal three hours later.

It appears from the combined results of all experiments that toxic levels of urea are reflected in the rapid rise of urea-N of the blood and an increase in blood $\text{NH}_3\text{-N}$ to values over 2.5 mgm. per cent. The extent of these blood changes with accompanying symptoms of alkalosis which may result in death is conditioned by the manner of administration. Apparently steers are able to consume in mixed feed relatively large quantities of urea, one steer receiving 400 grams daily; whereas, a similar or even smaller amount of urea suddenly directed into the esophagus or rumen may prove fatal.

SUMMARY

Oral administration of 40 grams of urea in water solution to a sheep under light anesthesia produced a rapid rise in urea and ammonia of the portal blood. Portal blood-ammonia values, which continued to increase during the two-hour observation period and reached 8.4 mgm. per cent, indicated hydrolysis of urea in the rumen and absorption of large quantities of ammonia. When adminis-

tered as a drench to steers, urea in amounts exceeding 100 grams produced a rapid rise in the levels of both urea and ammonia of the systemic blood. Ataxia appeared in steers when ammonia nitrogen of the systemic blood reached a level of approximately 2.5 mgm. per cent, and symptoms of alkalosis followed by death occurred at a level of about 4 mgm. per cent. When given as feed, mixed with other concentrates, urea in amounts up to 400 grams daily produced no ill effect in steers; in fact, it has been used successfully in this manner to supply part of the nitrogen (protein) needs of both sheep and steers in practical feeding trials (3).

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EFFECT OF THE KIDNEY OF THE FASTING RAT ON BLOOD SUGAR AND HEMOGLOBIN CONCENTRATIONS BEFORE AND AFTER EVISCERATION OR PARTIAL HEPATECTOMY¹

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IT HAS been found that the kidneys of the rat become an apparent source of blood sugar sometime after the animal's gastrointestinal tract between the lower esophagus and lower colon, pancreas, spleen and liver have been removed (1). The studies reported here were undertaken to determine how soon after evisceration this renal glucogenesis might be observed and whether or not the lesser degree of hepatic insufficiency induced by partial hepatectomy might also be adequate to reveal it. The study on the eviscerated preparation also afforded an opportunity to investigate the effect of faradic stimulation in the vicinity of the renal vein. This was done because the puncturing of the renal vein in taking blood had seemed to cause variations in the sugar concentration in successive samples (1). As a part of these studies, sugar estimations were made on a substantial number of pairs of samples drawn simultaneously from the aorta and renal vein prior to either evisceration or partial hepatectomy. These were examined statistically for evidence as to whether or not renal glucogenesis occurs in the fasting rat that has been subjected to no procedures other than the anesthesia and midventral incision necessary for taking the samples.

Hemoglobin analyses were made on all samples taken. It was assumed that the absolute amount of hemoglobin does not change as blood flows through the kidney and that therefore variations in its concentration can be used in estimating the hemoglobin concentration due to the renal excretion of water. The statistical analysis of the results indicated that this assumption may not be valid.

METHODS AND RESULTS

These studies were made on adult male, albino rats from 222 to 322 grams in weight that had been obtained from Sprague-Dawley as weanlings. They were allowed to fast for the four days prior to operation, for it had previously been found that renal glucogenesis is more readily observable in the fasting rat (2, 3). Tap water was given ad libitum. The eviscerations were performed as in preceding studies (1). The partial hepatectomies consisted of making a midventral incision

Received for publication April 5, 1948.

¹ Supported by a grant from the John and Mary R. Markle Foundation.

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into the belly, tying a single ligature around the attachments of the left and median lobes of the liver and excising the portion of the liver distal to the ligature. The blood samples were drawn and analyzed for sugar and hemoglobin as described previously (4). Great care was taken to draw the corresponding renal vein and aortic samples simultaneously and then analyze them in strict parallelism in order to avoid insofar as possible any systematic error that might effect one but not the other. The midventral incision into the belly was closed between samplings. Sodium amytal was used as the anesthetic (4).

The results of the studies made to determine how soon after evisceration renal glucogenesis might be observed are presented in table 1. The statistical analysis of these data shows that the concentration of sugar was significantly greater in the renal vein than in the aorta at the completion of the procedures about 9-16 minutes after the superior mesenteric and celiac arteries had been ligated. This increased concentration of sugar in the renal vein as compared with the aorta is also found to have been significantly greater than the statistically non-significant increase observed in the pre-evisceration samples in this group. The samples taken at about one and three hours after evisceration show the increased concentration of sugar in the renal vein as compared with the aorta in even more marked and significant degree. The incidental study on the effect of faradic stimulation in the vicinity of the renal vein supports the earlier impression that stimulation in this region might cause variations in the concentration of sugar found in that vein. In four of the five instances there was a marked increase and in the fifth a marked decrease in the observed concentration. These changes may have been secondary to changes in blood flow, for there was a definite diminution in the diameter of the renal vein after stimulation. The decrease in sugar concentration in the one instance may have been due to a back flow of blood into the renal vein from the vena cava occasioned by the withdrawal of the sample from the renal vein at a rate faster than could be supplied by a markedly reduced flow through the kidney.

The results of the studies made to determine whether or not partial hepatectomy might be effective in revealing renal glucogenesis are shown in tables 2 and 3. It was found that the concentration of sugar was significantly greater in the renal vein than in the aorta three hours after partial hepatectomy. The amount, percentage-wise, by which the sugar concentration in the renal vein exceeded that in the aorta is also found to be significantly greater than the percentage-wise smaller and statistically non-significant excess found previous to partial hepatectomy in this group. The results shown in table 3 indicate that there is no obvious advantage in studying renal glucogenesis 6 or 25 hours rather than 3 hours after partial hepatectomy.

The samples taken before either evisceration or partial hepatectomy indicate that on the average the concentration of sugar was higher in the renal vein than in the aorta even before the animals were subjected to these procedures. The higher concentration, however, proves to be statistically significant in only the group shown in table 3. Since the preliminary preparation of all the animals had been essentially similar, it was considered justifiable to combine all of the pre-evisceration and pre-partial hepatectomy samples for statistical study. This combined group then contained 45 pairs of renal vein and aortic sugar estimations. The renal vein sugar con-

TABLE 1¹

Animal	Samples taken 2-4 min. before evisceration				Samples taken 9-16 min. after evisceration				Samples taken 52-79 min. after evisceration				Samples taken 180-193 min. after evisceration				Samples taken 1 min. after 10 sec. faradic stimulation			
	Sugar		Hb.	[(Crv/Cao) -1]100	Sugar		Hb.	[(Crv/Cao) -1]100	Sugar		Hb.	[(Crv/Cao) -1]100	Sugar		Hb.	[(Crv/Cao) -1]100	Sugar		Hb.	[(Crv/Cao) -1]100
	Cao	[(Crv/Cao) -1]100			Cao	[(Crv/Cao) -1]100			Cao	[(Crv/Cao) -1]100			Cao	[(Crv/Cao) -1]100			Cao	[(Crv/Cao) -1]100		
1	90	.20	-2.19	-2.67	83	6.22	-2.67	-2.06	68	8.01	-2.06	-2.06	58	11.59	-1.49	-2.00	51	-20.00	-2.41	
2	93	2.76	-.20	.57	82	1.58	.57	1.53	64	5.58	1.53	1.53	56	25.50	-1.77	40.2	53	40.2	1.67	
3	80	9.27	1.62	.42	83	11.37	.42	.21	73	9.23	.21	.21	44	18.19	-4.38	36.55	45	36.55	-3.64	
4	92	5.37	.00	-1.16	81	10.25	-1.16	-2.37	64	9.79	-2.37	-2.37	44	21.38	.41	29.58	46	29.58	.00	
5	90	7.37	.67	-.75	77	10.88	-.75													
6	106	-5.93	-2.62	.00	100	3.32	.00	-1.00	79	9.39	-1.00	-1.00					48	73.7	-1.96	
7	89	4.74	.82	1.92	82	2.93	1.92	1.94	61	31.44	1.94	1.94	47	41.4	-.20					
8	86	-3.83	-2.00	.74	99	.74	-1.76	-2.00	73	13.49	-2.00	-2.00	49	31.9	.65					
9	78	6.16	-2.06	-1.96	78	11.91	-1.96	-1.23	52	31.08	-1.23	-1.23	55	43.6						
10	87	.42	-5.20	1.93	80	-1.15	1.93	3.14	60	10.53	3.14	3.14								
Mean	89	2.74	-1.12	-.35	84	5.80	-.35	-.25	66	14.28	-.25	-.25	50	27.7	-1.13	32.0	49	32.0	-1.27	
P		.1	.2-.1	.2-.1		.01-.001	.2-.1	>.9		.01-.001	.01-.001	.2		<.001		.2-.1		.2-.1		
P'						.02-.01				.01-.001										
Mean in mgm.		2.4				4.9				9.4				13.8				15.7		

¹ The number of minutes before or after evisceration or partial hepatectomy were counted from the time at which either the ligature around the celiac and mesenteric arteries or around the attachments of the left and median lobes of the liver was tied. 'Cao' and 'Crv' indicate concentrations in the blood taken from the aorta and renal vein, respectively. The aortic sugar concentrations are given in milligrams per 100 ml. A single analysis for hemoglobin 'Hb' was made on each sample. Most of the filtrates were analyzed in quadruplicate for sugar; the means of these analyses appear in the tables. The faradic stimulations were carried out within a few minutes after the previous samples had been taken. A Harvard Inductorium was used and it was set so that the stimulus was strong enough to cause a contraction when the electrodes were applied to the exposed psoas muscle.

The values shown in the row designated by *P* were determined by the method of testing for the significance of the differences between individual values in that column and the corresponding values for the samples taken before evisceration or partial hepatectomy. Values of 0.05 or less for *P* indicate that the mean shown in that column differs significantly from zero. Values of 0.05 or less for *P'* indicate that the mean shown in that column differs significantly from the corresponding mean of the samples taken prior to evisceration or partial hepatectomy. The row at the bottom of table 1 headed *Mean in mgm.* shows in milligrams per 100 ml. the amount by which the mean sugar concentration of the renal vein samples exceeded the mean concentration of the corresponding aortic samples.

centration for this group exceeded that of the aorta by a mean of 2.02 per cent with a standard deviation of 0.637 per cent. This gives a value of t of 3.17, which for a sample of this size indicates that the probability of obtaining this result by chance is less than 1 per cent.

The hemoglobin concentrations had a noticeable tendency to be lower in the

TABLE 2¹

Animal	Samples taken 1-6 min. before partial hepatectomy			Samples taken 170-190 min. after partial hepatectomy		
	Sugar		Hb	Sugar		Hb
	Cao	[(Crv/Cao)-1]100	[(Crv/Cao)-1]100	Cao	[(Crv/Cao)-1]100	[(Crv/Cao)-1]100
11	98	-.56	2.47	99	7.24	-2.28
12	86	1.93	1.42	131	3.84	.59
13	95	-6.92	1.04	71	16.45	1.86
14	96	2.86	.39	96	7.12	.97
15	102	.18	.82	99	2.58	-.79
16	77	3.94	1.04	69	7.30	-.91
17	82	3.38	.20	91	2.61	-1.92
18	85	1.95	-1.02	75	5.73	-.75
19	79	.70	-.62	68	-7.22	-5.12
20	122	4.45	-3.10	112	3.79	-1.69
21	112	-4.46	-.59	92	9.80	2.55
22	82	-9.47	.00	86	4.29	-1.42
23	89	8.28	.20	106	10.08	-1.35
24	73	11.59	-1.16	74	14.45	-.93
25	83	3.33	-1.52	90	2.86	-1.93
26	96	8.02	2.25	96	7.62	-1.65
27	91	.60	-1.73	117	-4.77	1.52
28	86	7.34	-.85			
29	99	.56	-1.95	102	.90	-2.40
30	89	2.28	.00	79	5.66	.99
31	90	.20	.84	79	2.40	-2.36
32	96	-1.72	1.67	89	8.46	-.37
33	82	.91	-.38			
34	105	-.35	-.22			
35	97	3.78	-4.19			
Mean	92	1.66	-.20	91	5.29	-.83
P		.1-.05	.6-.5		<.001	.05-.02
P ¹					.02-.01	

¹ See footnote to table 1.

renal vein than in the aorta. In one group shown in table 2 the difference is statistically significant. In order to study this finding further, all of the data pertaining to hemoglobin were combined for statistical analysis into a single group, which then contained 114 pairs of renal vein and aortic hemoglobin values. The renal vein hemoglobin concentration for the entire group was less than that of the aorta by a mean of 0.579 per cent with a standard deviation of 0.163 per cent giving a value of t of 3.55,

which for a sample of this size indicates that the probability of obtaining these values by chance is less than 0.1 per cent.

TABLE 3¹

Animal	Samples taken 2-5 min. before partial hepatectomy			Samples taken 361-369 min. after partial hepatectomy			Samples taken 1458-1512 min. after partial hepatectomy		
	Sugar		Hb.	Sugar		Hb.	Sugar		Hb.
	Cao	$\frac{[(Crv/Cao) - 1]100}{-1}$	$\frac{[(Crv/Cao) - 1]100}{-1}$	Cao	$\frac{[(Crv/Cao) - 1]100}{-1}$	$\frac{[(Crv/Cao) - 1]100}{-1}$	Cao	$\frac{[(Crv/Cao) - 1]100}{-1}$	$\frac{[(Crv/Cao) - 1]100}{-1}$
36	85	2.84	.64	83	-10.49	-.63	83	3.35	-2.28
37	79	-1.42	.42	80	3.27	-2.22	91	.00	-1.96
38	90	-2.86	.00	87	6.78	-.82	86	9.01	-.21
39	103	-.36	.20	83	1.33	-2.16	74	2.28	-5.21
40	120	5.44	2.89	82	7.61	1.66			
41	96	1.15	.40	92	2.98	.61	84	.44	.00
42	94	3.70	1.06	86	1.29	.82	92	2.59	-2.64
43	86	5.98	-1.67	87	-1.27	-.78	86	2.35	.00
44	76	5.39	.61	73	9.14	-3.15	73	7.62	.00
45	80	2.08	-.81	76	5.87	1.56			
Mean	91	2.19	.37	83	2.65	-.51	84	3.46	-1.54
P		.05	.4-.3		.4-.3	.6-.5		.02-.01	.1-.05

¹ See footnote to table 1.

DISCUSSION

Fermentation was not used in the analyses because it was expected to increase the variance and consequently the number of animals needed in each group. In previous studies (1, 3), however, the arteriovenous differences in sugar concentration for the kidney were not explicable on the basis of variations in a non-fermentable reducing substance.

Urine and lymph formation and variations in renal volume and sugar content may cause increases in the sugar concentration of the blood flowing through the kidney in the absence of renal glucogenesis. The possible magnitude of these increases must be considered if any interpretation is to be made of the relatively small percentage increase that was found for the group of samples taken prior to evisceration or partial hepatectomy. In this case the renal vein sugar concentration was a mean of 2.02 per cent higher than that of the aorta and the standard deviation of this mean was 0.637 per cent. By convention this mean is significantly greater than any arbitrary value which it exceeds by twice its standard deviation. It follows then that this observed increase is statistically significant of renal glucogenesis if the nonglucogenic processes mentioned cannot account for more than a 0.743 per cent increase, for 2.02 per cent exceeds this value by 2×0.637 per cent. If such an increase were to be accounted for by urine formation, the urine flow would have had to have exceeded 0.737 per cent of the blood flow. If the blood flow approximated normal at the time these samples were taken, this rate of urine flow would have exceeded 0.031

ml/100 cm.² of body surface/min. (7). This would have amounted to 185–235 ml/day for the size range of animals used. This approaches the maximum capacity of the kidney of the rat to excrete urine as found in studies on water intoxication (8). In a recent study (9) in which both urine flow and plasma flow were measured, the urine flow exceeded 0.737 per cent of the blood flow in only one group of animals. In this group a severe diuresis had been induced by the subcutaneous administration of normal saline and urea so that if the observed rate of urine formation had continued, rats averaging 149 grams in body weight would have excreted 120 ml. of urine per 24 hours. In the other groups, which exhibited less marked diuresis, the urine flow was substantially less than 0.737 per cent of the renal blood flow. Studies on another species, the dog, suggest that both the barbiturate anesthesia and puncturing of the renal vein present in these studies may have reduced both urine flow and renal blood flow (10–12). There is no evidence indicating that the reduction in blood flow would have exceeded the reduction in urine flow to such an extent that the latter would have exceeded 0.737 per cent of the former.

If it is assumed that the erythrocytes of the rat contain no sugar (13, 14) and that the renal capillaries are permeable to the blood sugar (15, 16), any increase in the plasma fraction of the blood in excess of 0.743 per cent of the arterial value would be adequate to account for the observed increase in renal vein sugar concentration. The renal blood flow, however, is so large when compared with the renal volume and carbohydrate content (17) that it seems improbable that any significant increase in renal vein sugar concentration can be accounted for by this mechanism. The same conclusion holds for the effect of the movement of sugar-free water into the intracellular space of the kidney, for this process like that of urine formation would need to have proceeded at 0.737 per cent of the blood flow through the kidney to account for the 2.02 per cent increase observed in blood sugar concentration.

In the dog, renal lymph flow probably exceeds that of urine (16); but since the sugar concentration in lymph approximates that in plasma, lymph formation can scarcely account for any significant increase in renal vein blood sugar concentration.

There is the possibility that the decreased concentration of hemoglobin observed in the renal vein as compared with the aorta may be a reflection of a difference in the degree of plasma skimming in the sampling procedure for the samples were drawn through No. 27 hypodermic needles. These needles have a bore of about 135 μ and it is conceivable that the renal vein blood may differ sufficiently from aortic in such properties as viscosity and the surface characteristics of the erythrocyte that there might be a significant difference in the proportions of cells and plasma drawn through the needle. If it is assumed that there has been no change in cell volume between aortic and renal vein blood, that the red cells contain no sugar and that the hematocrit is approximately 50 per cent, then the percentage decrease in hemoglobin will account for an equal percentage increase in blood sugar. If the increases in blood sugar observed in the samples taken before evisceration or partial hepatectomy are corrected on this basis by subtracting the decreases observed in hemoglobin concentration, and if the results are analyzed statistically it is found that there remains a

mean increase of 1.774 per cent in renal vein sugar concentration with a standard deviation of 0.600 per cent giving a value of t of 2.54 which for samples of this size indicates a value of $P < .02 > .01$. This sort of plasma skimming due to the use of small bore needles therefore does not account for the observed increase in blood sugar.

Consideration then of the various possible non-glucogenic mechanisms reveals that either singly or collectively they would have had to have proceeded at rates so great as to be unlikely in order to account for the observed increase in renal vein sugar concentration. This supports the interpretation that the 2.02 per cent increase in renal vein sugar concentration prior to evisceration or partial hepatectomy indicates that renal glucogenesis was present at that time and therefore is probably of importance in the physiology of the fasting rat.

The increases in blood sugar in the renal vein as compared with the aorta observed after evisceration and three hours after partial hepatectomy are too large percentage-wise to be explained on any basis other than renal glucogenesis even though there probably were large reductions in blood flow—especially after evisceration. These increases became progressively larger after evisceration, not only percentage-wise, but also in terms of milligrams of sugar per 100 ml. It is also notable that the increase in renal vein sugar observed in the first post evisceration sample and in the sample taken three hours after partial hepatectomy occurred in the absence of any marked reduction in arterial sugar. Hypoglycemia, therefore, does not seem to be a factor in increasing the excesses of sugar found in the renal vein as compared with the aorta.

The study on the effect of faradic stimulation in the vicinity of the renal vein serves to emphasize the uncertainties present concerning the probable variations in renal blood flow. Until these are in some way accounted for, it is impossible to interpret arteriovenous differences such as were studied in these experiments in terms of rates of glucogenesis. It has, however, been shown that increased concentrations of pyruvate, lactate, alanine, fumarate, succinate and maleate increase the carbohydrate synthesis in kidney slice preparations (18, 19). Since the blood concentrations of at least some of these rise in acute hepatic insufficiency, these findings suggest that there may be an increase in renal glucogenesis after evisceration or partial hepatectomy.

The lower concentration of hemoglobin in the renal vein as compared with the aorta can scarcely be explained on the basis of plasma skimming (20, 21) because of the very high rate of blood flow characteristic of the kidney. The same consideration also seems to exclude the renal excretion of hemoglobin from hemolyzed red cells. Some reports by Bingold (22) suggest that the kidney may be a special site of hemoglobin metabolism. This seems almost incredible as an explanation for the observed rate of hemoglobin disappearance in our studies is of a different order of magnitude from that accounted for by any other studies on hemoglobin metabolism. There remains the possibility of a sort of artificial plasma skimming due to the use of small bore needles in sampling as was discussed apropos the significance of the increase in renal vein sugar. This explanation it must be confessed also seems rather unlikely.

SUMMARY

A significant increase in the blood sugar in the renal vein as compared with the aorta was found in the rat that had been allowed to fast for four days. This increase became even more marked after evisceration or partial hepatectomy.

The hemoglobin concentration was found to be significantly lower in the renal vein than in the aorta.

The authors express their appreciation to Miss Lucille Snyder for her assistance in making the analyses for blood sugar.

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THE KIDNEY AND NITROGEN METABOLISM

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CONTRASTING results of bilateral renal artery ligation and nephrectomy have been reported. They involve nitrogen catabolism as indicated by the blood nonprotein nitrogen and include symptomatology, survival time and the hemorrhagic-necrotizing lesions of blood vessels and heart. Functional and structural changes associated with tying the renal arteries are reproduced by the intravenous injection of protein-containing extracts of kidney into nephrectomized dogs. The hypothesis follows that specific kidney substances, probably proteins or their derivatives, are directly or indirectly responsible for these effects. Efforts to isolate such substances from autolyzed and fresh kidney of both homologous and different species and from other organs have been rewarding (1) and are still in progress. The hypothesis that the increase of nitrogen catabolism following injection of renal extracts might be associated with the production of toxic protein intermediates suggested a more direct approach. This involved administration of large amounts of protein both to animals with reduced kidney function and after bilateral nephrectomy. Preliminary qualitative studies were extended to include quantitative determination of nitrogen intake and output as well as of total urea formation. The results of this investigation, indicating that the kidney is involved in nitrogen metabolism, are included in the report that follows.

MATERIALS AND METHODS

The reduction of kidney function (2-6) was accomplished in several ways including ligation of branches of the renal artery, wedge or pole removal, injection of sodium linolenate into the renal artery and the application of heat. The first two of these methods require no comment; the third has been reported recently (7); only the last of these techniques will be described.

The kidney is exposed through a flank incision. After the application of a rubber-shod clamp to the pedicle, it is placed into a specially-prepared double-walled rubber envelope. Two short rubber tubes serve as inlet and outlet for a flow of water from a bath kept at 48°C. After 30 minutes perfusion the envelope and clamp are removed, the kidney replaced in the abdomen and the incision closed. The second kidney may be removed immediately after the heating of the first or subsequently.

Received for publication February 19, 1948.

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¹ Aided by a grant from the Commonwealth Fund.

All operative procedures were carried out with aseptic technique and under nembutal anesthesia. Explantation of the left kidney under the skin was accomplished after the method of Rhoads (8). Inulin was used for determination of glomerular filtration, diodrast for renal blood flow and maximal tubular excretory capacity and glucose for maximal tubular reabsorptive capacity (9, 10).

The urine was collected over 24-hour periods in flasks contained in ice-filled insulated boxes. The nitrogen was calculated for gelatin; for meat it was determined by the Kjeldahl method; the amount of the weighed food not consumed was recorded. Each experimental period involved several days' study of nitrogen balance on kennel diet, preceding the high protein feeding. Intrajejunal administration of a protein hydrolysate (Amigen²) was accomplished through a short midline incision, injecting the material into the gut with a syringe and a 15-gauge needle. This required approximately 10 minutes. The needle was then withdrawn, the intestine replaced and the abdomen closed. All nitrogen determinations of urine or blood were carried out by the half-micro Kjeldahl method. The tungstate filtrates of Folin-Wu (11) were used for the nonprotein nitrogen and the urease-aeration method of Van Slyke *et al.* (12) for the urea determination in urine and blood. The total urea formation was calculated from the 24-hour urea content of the urine and from the increase in the plasma urea above the level of the pre-feeding period. The maximal value for the distribution of urea in body water, 75 per cent of the body weight, was used (see footnote to dog 5, table 3).

For nephrectomized animals urea formation was calculated from the urea nitrogen level of the plasma determined repeatedly after feeding. The normal increase in urea nitrogen in fasting nephrectomized dogs has been found to be between 2 and 3 mgm. blood (13) per hour; a value of 2.5 mgm. per cent per hour, therefore, was deducted. The difference between the urea N value after feeding and after fasting was the basis for the calculation of the total urea-N formation. The plasma amino nitrogen was determined according to the method of Frame, Russell and Wilhelm (14, 15).

RESULTS

Nitrogen balance in normal dogs. In one five- and one three-day period of heavy meat feeding, normal dogs 1 and 2 established an almost complete nitrogen equilibrium within 24 hours. This prompt adjustment has long been known (16).

Nitrogen balance in dogs with reduced kidney function. The results of 10 feeding experiments performed on 9 dogs with reduced kidney function follow.

DOG 3. Removal of the right kidney, five days after the application of heat to the left, was followed by a transient anuria and marked elevation of the blood NPN. After two weeks these signs of acute kidney insufficiency subsided. The damage to the left kidney was permanent as evidenced three months later by polyuria and great rises in NPN, following heavy meat or gelatin feeding. Clearance determination at such time showed a reduction of kidney function to 20 per cent of normal. Despite the low clearance the dog did not seem ill, ate kennel food regularly and did not lose

² Mead, Johnson & Co. Evansville, Indiana.

weight. Two nitrogen metabolism studies, with an interval of two weeks, were carried out nine months after heating the left kidney. The result of the first follows. The N-intake in 6 days was 146.5 grams, urea-N in urine in six days was 60.41 grams, and urea-N in body fluid was 0.3 gram. The percentage of the N-intake not transformed into urea was 58.5. The second study is tabulated below:

DOG 4. Heating the left kidney and removal of the right reduced renal function to 35 per cent after four months. Four operative procedures at intervals of one month, involving three successive branch ligations of the renal artery and amputation of both poles of the kidney, were required to reduce kidney function to 20 per cent. The animal remained very lively, ate kennel food regularly and maintained its weight. The results of a four-day feeding experiment with a gelatin hydrolysate four months after the last operation are contained in table 2.

TABLE 1. HIGH MEAT INGESTION AND NITROGEN METABOLISM IN A DOG WITH REDUCED KIDNEY FUNCTION

Dog 3	Diet	Urine			Blood NPN
		Total N	Urea-N	Amount	
Days					
0-1	Kennel diet	3.349	3.035 gm.	79 ml.	34.2 mgm.%
1-2	Boiled meat 2 lbs = 28.5 gm. N.	13.27	10.819	750	128.8
2-3	"	18.1	16.07	1035	127.7
3-4	"	16.71	14.954	1010	138.9
4-5	"	16.22	14.525	860	120
5-6	"	13.99	12.094	748	153
6-7	"	18.5	16.66	1000	114
7-8	Kennel diet	5.89	5.35	275	22

N intake in 7 days was 174.35 grams; urea-N in urine in 7 days was 90.472 grams; urea-N in body fluid, 0; N not transformed into urea, 48.1%.

DOG 5. Renal function was reduced to 18 per cent of the normal by removal of the right kidney, heating the left and branch ligation of the renal artery. Despite persistent elevation of the blood NPN (70-75 mgm. per cent) during the four months after the last operation, the animal was lively and maintained its weight.

A three-day feeding of hydrolyzed gelatin was then carried out. The results follow.

DOG 6. Right nephrectomy, heating of the left kidney and final ligation of renal artery branches reduced the kidney function to 18 per cent. This was reflected in the permanent elevation of the blood NPN (92 to 150 mgm. per cent). The animal nevertheless appeared perfectly healthy, was especially lively and regularly ate the usual amount of kennel food. Two months after the last operation a meat-feeding experiment was undertaken, with the following results. The nitrogen intake in three days was 85.5 grams, the urea excretion, 37.351 grams and the urea-N contained in body fluid, 18.1 grams³; 35.1 per cent of the nitrogen intake was not transformed into urea.

³ See footnote to dog 5.

Proffered meat was eaten rapidly on the first and second days. On the third, a much longer time was required, and all food was rejected thereafter. There was slight vomiting on the fifth day with evident and progressive apathy. Anuria and a blood NPN of 457 mgm. per cent on the sixth day were shortly followed by death.

TABLE 2. HIGH GELATINE INGESTION AND NITROGEN METABOLISM IN A DOG WITH REDUCED KIDNEY FUNCTION

Dog 4	Diet	Urine			Blood NPN
		Total N	Urea-N	Amount	
Days					
0	Kennel diet	5.285 gm.	3.70 gm.	775 ml.	40.6 mgm.%
1	90 gm. gelatin; tryptic hydrolysate 16 gm. N.	9.97	8.410	690	89
2	180 gm. gelatin; tryptic hydrolysate 32 gm. N.	16.12	13.765	1280	128
3	" "	13.23	13.194	885	132.7
4	90 gm gelatin; tryptic hydrolysate 16 gm.	10.53	8.863	720	132.0

N intake in 4 days was 96.0 grams; urea-N in urine in 4 days, 44.23 grams; urea-N in body fluid, 6.12 grams; total urea-N, 50.35 grams; N not transformed into urea, 47.55%.

TABLE 3. HIGH GELATINE INGESTION AND NITROGEN METABOLISM IN A DOG WITH REDUCED KIDNEY FUNCTION

Dog 5	N Intake	Urine		Blood NPN
		Urea-N	Amount	
Days				
0	3.6 gm.	3.0 gm.	400 ml.	73 mgm.%
1	26	14.2	1340	201
2	26	12.1	1456	242
3	26	12.4	1240	213 ¹
4	—	—	300	370

Total N intake, 78 grams; urea-N excreted, 38.7 grams; urea-N in body fluid, 10.5 grams; total urea-N, 49.2 grams; N not transformed into urea, 37%. The dog died on the fifth day.

¹ The calculation for urea-N in body water is based on the assumption that total body water equals 75% of the body weight. Lower values reported for distribution of urea in body water would increase the percentage of nitrogen not transformed into urea, (17, 18).

In subsequent experiments, the three days' heavy protein diet was reduced to two.

Repeated ligation of renal artery branches and excision of kidney tissue reduced clearances to 20, 24, 19, and 21 per cent of normal, respectively, for dogs 7, 8, 9 and 10. They remained in excellent condition. Extensive damage to the renal epithelium was produced in no. 11 by injection of sodium linolenate into the renal artery. The dog remained in a suburemic state for three weeks (NPNs between 112 to 196

mgm. per cent). Kidney damage was evident from the then persistent elevation of the NPN (66-78 mgm. per cent).

The results of two-day meat feeding of *dogs 7, 8, 9, 10 and 11* carried out from four to seven months after stabilization of their renal function are tabulated in table 4.

Nitrogen balance in nephrectomized dogs. To reduce the shock of operation and resulting loss of appetite the usual one-stage operative procedure under nembutal anesthesia was abandoned. Right nephrectomy and explantation of the left under the skin was followed in about 10 days by the removal of the left kidney. This was accomplished expeditiously with the aid of local anesthesia. All three animals ate offered meat immediately after operation and two of the three ate a second portion three hours later. Calculations of urea formation were based upon values of blood urea determined 30, 48 and 72 hours after nephrectomy.

The marked impairment of urea formation demonstrated in the three nephrectomized dogs after meat-feeding confirms the similar observations on dogs with reduced kidney function. Figure 1 represents a survey of the results so far obtained.

TABLE 4. HIGH MEAT INGESTION AND NITROGEN METABOLISM IN FIVE DOGS WITH REDUCED KIDNEY FUNCTION

Dog	N intake	Total urca-N formation	Percentage of N not transformed into urea
7	57.0 gm.	39.71 gm.	30.4
8	57.0	43.81	23.2
9	57.0	37.6	34.1
10	57.0	39.6	30.6
11	57.0	32.3	43.4

The three dogs exhibited toxic symptoms as early as six to eight hours after feeding, as exemplified by loss of appetite, weakness and listlessness that went on to coma.

Similar toxic symptoms following meat feeding were present also in the dogs with reduced kidney function. They persisted for days after the cessation of the experiment, even when there was ultimate recovery, and were progressively more severe until death in *dogs 5 and 6*. There can be little question concerning the relation of meat ingestion and toxic symptoms and it would seem that these can be attributed to inadequate protein catabolism and accumulation of intermediates. If this interpretation can be substantiated, a link will have been supplied with several recently uncovered facts. These pertain to the potentiation of epinephrine by particular protein intermediates (19) and also possibly to the vascular lesions recently produced with allylamine (20).

Toxic effects of intrajejunal feeding of a protein hydrolysate to nephrectomized dogs. Amigen was used to ascertain the significance of protein split-products independent of the effects of the potassium content of meat. It was given by direct intrajejunal injection in amounts corresponding to the protein content of one and one half to two pounds of meat. The reaction of six normal and six nephrectomized dogs to large amounts of Amigen (10 to 17 gm/kgm.) differed strikingly. This mixture of amino

acids and peptides, only transiently and insignificantly toxic for normal dogs, proved to be harmful for nephrectomized animals. They rapidly developed coma that persisted until death. The survival times of 17 to 40 hours compared unfavorably with the average of 100 hours for fasting, nephrectomized dogs.

TABLE 5. UREA FORMATION IN THREE NEPHRECTOMIZED DOGS AFTER INGESTION OF HEAVY MEAT MEALS

Dog no.	N intake	Blood withdrawn after feeding	N-values		Percentage of N not transformed into urea
			Blood urea-N	Total urea N formed	
12	49.87 gm.	30 hrs.	220 mgm. %	16.24 gm.	67.5
		48	283	20.00	59.7
		72	291	16.34	67.3
13	22.0	30	243.7	10.20	53.7
		48	277	11.02	50.0
		72	336	12.28	55.8
14	42.75	30	175	10.13	76.4
		48	230	11.4	76.3
		72	256.2	10.10	73.4

See footnote to dog 5.

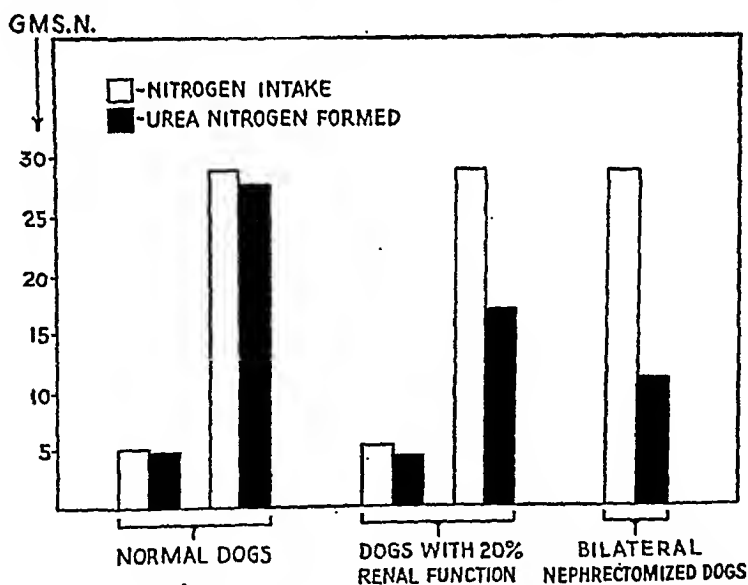


Fig. 1. COMPARISON OF NITROGEN METABOLISM of dogs with normal, reduced and eliminated renal function.

Not more than 10 per cent of all the ingested amigen could be recovered from the urine of the normal dogs, in successive collections extending over the few hours such animals displayed symptoms; their resistance therefore cannot be attributed to renal excretory function.

The observed effects were assumed to be caused either by all or by one or more of the specific components of the amigen. Accordingly it was planned to substitute individual amino acids for the protein hydrolysate. This has now been accomplished with glycine, chosen on the basis of the relation of its metabolism to kidney tissue (21, 22).

Table 6 shows the results of glycine feeding in four normal and six nephrectomized dogs: four of the six nephrectomized dogs died within one and one half hours after the administration of glycine. In the sixth, the expected survival time was halved and in the fifth it was reduced by one third. Nitrogen determinations in the urine of the normal dogs indicated the excretion of less than 10 per cent of the introduced glycine during four hours after feeding.

The level of plasma amino nitrogen, as can be seen in table 6, does not seem to be

TABLE 6. RESULTS OF GLYCINE FEEDING TO NORMAL AND TO NEPHRECTOMIZED DOGS

	No.	Glycine	Plasma Amino-N; hours after feeding					Surv. Time
			0 hr.	1 hr.	2 hr.	3-5 hr.	6-8 hr.	
Normal dogs	26	5 gm/kgm.	5.6	39.8	52.8	14.6	8.6	Lived
	27	5	5.5	30.6	28.1	8.1	6.6	"
	28	5	6.0	39.1	27.8	9.1	6.2	"
	29	2.5	4.7	38.0	26.3	8.1		"
Nephrectomized dogs	30	5	10.4*	46				1 hr.
	31	5	8.3*	42				1½ hr.
	32	5	6.0					¾ hr.
	33	2.5	5.85	57.7				1 hr.
	34	2.5	4.5	28.0	18.5	5.7		4 days
	35	2.5	12.7*	48.4	49.3	23	19	2½ days

* Feeding time, i.e., '0 hour' was 24 hours after nephrectomy in dogs 30, 31 and 35. Their plasma amino-N before nephrectomy was 4.5, 5 and 5.25 mgm. %.

related to the mechanism of the toxicity of glycine for nephrectomized dogs. Its harmlessness for normal dogs, even though only a small fraction appears in the urine, suggests a possible detoxifying, metabolic function for the renal tissue. Further studies with other amino acids are planned.

DISCUSSION

Urea formation and elimination of nitrogen in an adult, healthy, well nourished animal corresponds closely to the nitrogen intake. When the normal diet of such animals is supplemented with large quantities of protein, nitrogen equilibrium is restored within 36 hours (23). Significant positive nitrogen balance with adequate protein intake occurs under special conditions, including growth, protracted undernourishment and marked and prolonged increase in muscular activity (24).

Advances in endocrinology involving particularly the anterior pituitary, adrenal, pancreas and gonads have provided a new approach to problems of nitrogen metabolism. Anterior pituitary extracts containing the growth hormone increase the

anabolism of nitrogen in normal animals (25, 26, 27). After nephrectomy, this is reflected by a decrease in the blood nonprotein nitrogen (28). The growth hormone containing factor of the anterior pituitary further augments the significant nitrogen retention that follows adrenalectomy (29). Nitrogen retention has recently been shown to follow administration of androgens (30). The fact that the anabolic action of anterior pituitary is only demonstrated in the presence of adequate amounts of insulin (31, 32) emphasizes the importance of this hormone in nitrogen metabolism.

Several of the examples of anabolic stimulation so far cited represent protein synthesis as demonstrated by analysis of the carcass (33, 34). Other possibilities must be considered for positive nitrogen balance presented in this report, including the results of high nitrogen intake by animals with reduced or eliminated renal substance. Reduction in urea formation as demonstrated by these animals may represent incomplete nitrogen catabolism with accumulation of intermediates that the organism is incapable of carrying to the usual non-toxic end product. Such a hypothesis would support the possibility that protein intermediates are causatively associated with the toxic symptomatology these animals manifest. It will be recalled that damaging influences of high protein diets on rats with their renal function reduced by amputation (36) or nasugi nephritis (37) have been reported previously. The hypotheses also are in keeping with two recently demonstrated facts referred to earlier in this report: the production with allylamine of lesions of the heart and blood vessels, indistinguishable from those that follow renal artery ligation in animals and those associated with malignant nephrosclerosis in man (20), and the dependence of the potentiation of epinephrine upon specific protein intermediates (19).

It has been shown that the well-known transformation of citrulline to arginine by the liver (35) is duplicated by the kidney (21). This suggests the possibility of complementary action by these two organs under the stress of increased nitrogen catabolism. With impaired or eliminated kidney function, the availability of arginine to the liver for ornithin formation may not be adequate and this may be expressed by acceleration of the urea cycle as demonstrated by liver arginase activity. Evidence so far obtained is consistent with this hypothesis.

SUMMARY

Dogs with kidney function reduced to 17 to 24 per cent of normal remained in good health and maintained their weight for many months on kennel food despite a constant elevation in blood nonprotein nitrogen. Replacement of kennel food by a diet rich in meat or protein for two to six days resulted in 30 to 55 per cent urea nitrogen formation below the nitrogen intake. This deficiency in urea formation was still more marked, 50 to 76 per cent of the nitrogen intake, in nephrectomized dogs on protein rich diet. Marked signs of toxicity followed ingestion of the high protein diet in nephrectomized animals and those with reduced renal function. Intrajejunal administration of amigen in normal dogs, in amounts corresponding to a high protein diet, was followed by transient mild toxic symptoms; the same procedure resulted in significant reduction in the survival time of nephrectomized animals. When glycine

replaced intrajejunal administration of amigen, differences in the response of normal and nephrectomized animals were even more pronounced.

Technical assistance was given by Miss Helen Criscuolo, Mr. Edward Iannucci, and Mr. Peter Integlia.

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FLUID SHIFTS DURING EXPOSURE TO ACCELERATIONS: RAPID LOCAL CHANGES UNDER NEGATIVE G¹

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THE filtration of fluid from the circulation under conditions of increased intravascular pressure has been a consistent finding by many workers. In man the change from a reclining to a standing posture causes a significant increase in the intravascular pressure in the legs (1). This in turn leads to an increase in leg volume, which is due in part to the accumulation of interstitial fluid (2-4). The accumulation of fluid is accompanied by a significant hemoconcentration of the venous blood draining the legs (5). The total fluid shift is of sufficient magnitude to cause a measurable hemoconcentration of the entire blood volume (6, 7). A similar shift of fluid from the blood to the tissue spaces results from venous occlusion, even at low venous pressures (8, 9, 4). Several recent studies (10-12) demonstrate a simultaneous increase in both the arterial and venous pressures in the stressed end of animals exposed to radial acceleration. There should therefore be an increased hydrostatic pressure within the capillaries, and an outward filtration of fluid would be expected.

A small but easily measurable fluid loss from the circulation of men exposed to 'positive g' for periods of 3-5 minutes has been reported. In these studies the fluid loss was determined from the increased hematocrit of blood samples taken from the veins of the subject's arm (13). Dogs show a much larger fluid loss after 6.2 g for 1-5 minutes (14).

More recently at this laboratory gross evidence of filtration has been demonstrated during the autopsy of animals subjected to negative g. The significant findings have included 1) tongue swollen, edematous and extruding from the oral cavity; 2) ears thick, turgid and tending to lose their cartilaginous and venous markings; 3) mucosal surfaces of the larynx edematous, frequently causing strangulation; and 4) loose tissues of the face and neck markedly edematous. We obtained more direct indications of a shift of fluid from the intra- to the extra-vascular compartment, during negative g, from occasional hematocrit and plasma protein determinations on the blood of animals primarily under investigation for other effects of acceleration. As animals subjected to consecutive exposures to negative g gave results which were

Received for publication March 9, 1948.

¹ This study was supported in part by Contract No. N6ori77, Task 1, between the United States Navy and the University of Southern California.

² Lt. (MC) USN. The opinions expressed are those of the authors and do not indicate the opinion of the U.S.N.

irregular and at times conflicting, present studies have been limited to the fluid shifts from a single exposure.

METHODS

Twelve experiments were performed on 6 rabbits (*experiments 1-6*) and on 5 goats (*experiments 7-12*). Nembutal anesthesia was employed in all cases except for the first 3 rabbits. In these no anesthesia was considered necessary, as sampling was from the ear veins.

All animals were exposed to acceleration on the 'human centrifuge' of the Department of Aviation Medicine at the University of Southern California. The effective radius of the centrifuge arm at the animal position varied from 13 to 15 feet, and the required g ($1 g$ = acceleration of gravity) was attained by varying the speed of rotation. Under g the effective weight of all tissues increases by the multiple of the g force applied. The experiments were conducted with the long axis of the animal parallel to the radius of the centrifuge and the head of the animal directed toward the periphery

TABLE I

ANIMAL	MAGNITUDE OF G	BLOOD SOURCE	HEMATOCRIT			PLASMA PROTEIN			X FLUID LOSS cc/100 cc BLOOD	Pr PROTEIN LOSS GM/100 cc BLOOD	Pr X (PROTEIN) GM/100 cc FIL- TRATE
			t max. (sec.)	Maximum as % of control	Level after 5' as % of control	t max. (sec.)	Maximum as % of control	Level after 5' as % of control			
R14	-8	ear vein	82 ¹	109.3	99.4	82 ¹	105.9	98.4	8.5	.39	4.6
R15	-8	"	78 ¹	109.0	102.9	78 ¹	105.4	99.4	7.5	.27	3.6
R16	-5	"	241	101.7	100.2	56 ¹	101.4	97.2	1.7	.04	2.4
R17	-5	common jugular	69 ¹	105.6	99.2	69 ¹	102.8	100.0	5.3	.23	4.3
R18	-5	"	146	108.9	105.0	138	95.8	92.0	8.2	.67	8.2
R20	-5	"	59 ¹	105.8	101.4	59 ¹	105.4	100.8	5.5	.14	2.5
GL3	-5	"	80	118.8	110.4	80	102.5	101.1	15.8	.98	6.2
GL4	-5	"	48 ¹	118.5	110.5	48 ¹	106.3	99.6	15.6	.95	6.1
GL5	-5	"	92	128.0	107.6	92	109.9	99.5	21.9	1.26	5.8
GL7	-5	"	120	109.1	97.4	120	100.4	97.2	8.4	.70	8.3
GS1	-5	"	90	122.0	121.4	60	113.5	108.2	18.0	1.07	5.9
GS1(2)	-5	"	25 ¹	112.3	100.0	45	110.3	101.1	10.9	.67	6.1
Goat Averages			76	118	108	74	107	101	15.1	.94	6.4

¹ First post-run sample obtained.

(negative g in that the accelerative force acts in the axis from head to feet). *Experiments 1 and 2* (see table 1) were run at $-8 g$. The findings of petechiae and frank hemorrhages indicated that this force was too great to permit study of fluid shifts without the complicating factors of gross vascular injury and blood loss. Therefore, all subsequent experiments were run at $-5 g$. The duration of exposure at peak g was 30 seconds for the rabbits and 15 seconds for the goats, giving a stress of 4.0 and 2.5 g minutes to the two rabbit groups and 1.25 g minutes to the goats.

In all experiments a control blood sample was taken before the animal was exposed to acceleration. The post-run samples were obtained from the same vessels as were the controls, as soon after the run as venapuncture could be accomplished. The time interval from the stopping of the centrifuge was noted for each sample. To expedite the post-run sampling, a single puncture was made and successive samples were taken through the same needle into fresh 2 cc. syringes moistened with a minimum amount of heparin. The blood samples were transferred to Wintrobe hematocrit tubes and centrifuged for 30 minutes at 3000 r.p.m. Following the determination of hematocrit values, the supernatant plasma was pipetted off and used for protein determination by the falling-drop method (15).

The locations for sampling were veins in or draining that portion of the animal subjected to maximum force (in the case of the negative g , the head end). In *experiments 1, 2 and 3* ear veins were used. The restricted circulation in the ear, however, made for difficulty in obtaining rapid samples and introduced other unnecessary errors. Therefore subsequent sampling was from the jugular vein.

The change to goats as experimental animals was prompted for several reasons, related primarily to the size and weight of the animal. One was the ease of obtaining puncture samples from the jugular vein. Also the large total blood volume minimized the hemorrhagic effects and thus contributed to greater accuracy. Furthermore, the presence of many other easily available vessels throughout the body makes possible comparative studies on the resultant filtrations from variously affected portions of the animal.

The goat in *experiment 11* was re-used one week later for *experiment 12*. During *experiment 11* the animal lost much fluid as a result of salivation and emesis which are common with goats under nembutal anesthesia and negative g . During the interval week, although there was no indication of

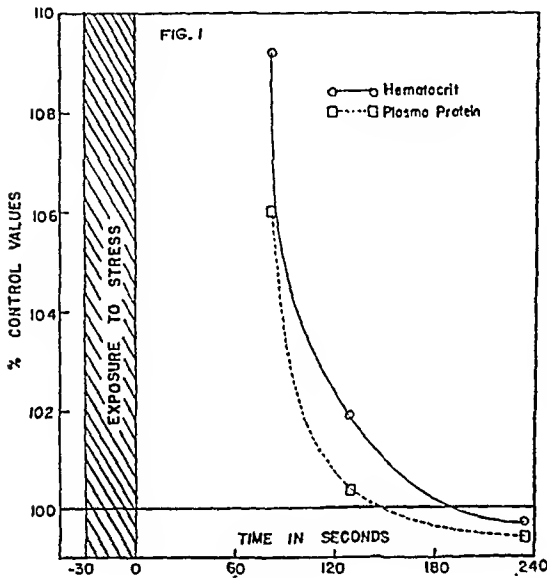


FIG. 1

Fig. 1. HEMATOCRIT AND PLASMA PROTEIN CONCENTRATION CURVES AFTER EXPOSURE OF RABBIT TO -5 G FOR 15 SECONDS.

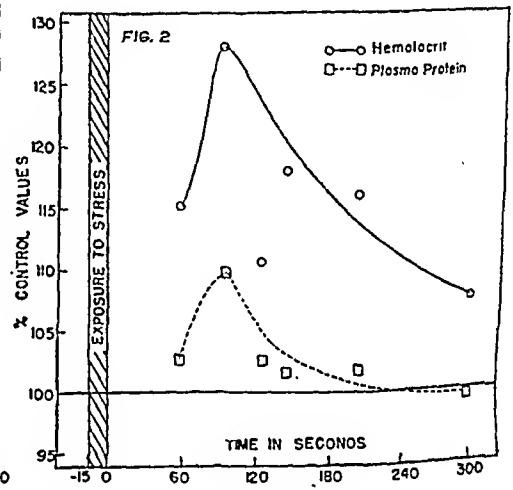


FIG. 2

Fig. 2. HEMATOCRIT AND PLASMA PROTEIN CONCENTRATION CURVES AFTER EXPOSURE OF GOAT TO -5 G FOR 30 SECONDS.

local infection from the vessel exposures, the animal apparently failed to restore its normal hydration. It died one hour after the run of *experiment 12* in respiratory failure. The other animals were used for acute studies on other problems after the experiments here reported had been concluded.

CALCULATIONS AND RESULTS

The results of these experiments suggest that high g forces lead to an extreme but transient concentration of both erythrocytes and plasma proteins. Calculations based on the data obtained suggest that the fluid loss is accompanied by an increased permeability of the vascular wall to proteins. Although the data may not yet be considered quantitatively accurate, they are sufficiently consistent to permit qualitative summary. The significant data are presented in table 1 as follows. The time given is the number of seconds between the end of the run and the time at which the

maximum hematocrit and plasma protein ratios were noted. Hematocrit and plasma protein concentrations are expressed as per cent of the control values. Their ratios at maximum concentration and five minutes after the runs are given. The maximum values were attained in the goats in about 1-1½ minutes after the end of the run. In some cases the maximum values noted were also the first samples taken after the run (see fig. 1). With improved technique a sufficient number of points has been obtained on the ascending portion of the curve to justify the conclusion that jugular blood samples show increasing concentration for a short period after the run and then become more dilute (see fig. 2). The average of six experiments on goat jugular-vein blood showed a hematocrit peak of 118 per cent of the control and a plasma protein peak of 107 per cent.

Plasma protein peaks occurred at about the same time as do those of the hematocrit, but were not as high. The return of plasma protein concentration to the normal control levels was more rapid than in the case of the hematocrit and in some experiments the values at five minutes were significantly below the pre-run levels.

At the same imposed *g* force, rabbits showed lesser tendency to concentrate than did goats. Rabbits showed a tendency to concentrate more when run at high *g* forces, e.g., -8 *g* as against -5 *g*.

Included in this table are estimates of the amount of fluid lost and the amount of protein lost per 100 cc. original blood, as calculated for the control and peak data, according to Landis' equation (8). A ratio of these two figures gives the protein concentration of the filtrate. This averages, in the goats, to 6.3 grams per cent or 82 per cent of the original plasma concentration.

DISCUSSION

According to the classic Starling hypothesis (16), an increase in the intravascular hydrostatic pressure, all other factors remaining constant, should result in an increased filtration of fluid from the circulation. Abundant evidence exists of decreases in the circulating blood volume or local hemoconcentration under conditions of generalized and local increases in venous and capillary hydrostatic pressures. In many cases the kinetics of fluid transfer has been studied and found to conform to the general requirements of the hypothesis (9, 17). The accumulation of fluid in the extravascular tissue spaces tends to increase the tissue pressure, and thus serves to diminish further fluid loss (18-20).

Under conditions of negative *g* the effective hydrostatic pressure in the capillaries of the head must be significantly above normal. Of the other factors involved in regulating the rate of fluid transfer across the capillary wall, only the tissue pressure might show rapid changes. Since it is unlikely that there is a continuous column of fluid in the tissues with dimensions similar to the vascular column, an increase in the tissue pressure of sufficient magnitude to off-set the change in the intravascular pressure seems improbable. The changes in tissue pressure appear to result indirectly from the postural changes, and follow slowly the changes in venous pressure (21). The increase in colloid osmotic pressure of the plasma which results from the early loss of fluid from the blood becomes relatively unimportant during the period of stress in the light of the marked protein leakage across the vascular wall. This same

protein leakage would tend to minimize the effect of changes in the colloid osmotic pressure of the tissue fluid. The net effect of negative g on fluid balance should, therefore, favor rapid outward filtration in the stressed areas.

Under negative g samples taken from the common jugular vein afford a fairly accurate sampling of the blood draining the area which has been subjected to maximum stress. An estimate of the effective hydrostatic pressure in the stressed areas may be made from figures given in table 2. The data are taken from Lombard (12) and represent the values of carotid and jugular pressures during the run for which the hemoconcentration data are here reported. In most cases the calculated arteriovenous pressure difference is less during the run than in the same animal before or after. This lowered pressure difference would suggest stagnation, or at least diminished blood flow in the head.

TABLE 2
Vascular pressures in mm. Mercury

		BEFORE ACCELE- RATION	AT END OF ACCELE- RATION ¹	AFTER 5" OF g	AFTER 10" OF g	AFTER 15" OF g	AFTER STOP OF CENTRIFUGE	5" AFTER STOP
Lombard	Carotid	79	212	203	201	200	120	100
Goat 3	Jugular	7	98	150	147	153	19	11
	Difference	72	114	53	54	47	101	89
Lombard	Carotid	72	166	218	210	207	70	70
Goat 4	Jugular	17	163	158	158	158	20	19
	Difference	55	3	60	52	49	50	51
Lombard	Carotid	91	265	256	273	279	101	104
Goat 5	Jugular	-3	70	123	128	126	1	1
	Difference	94	195	133	145	153	100	103
Lombard	Carotid	101	239	244	243	240	101	110
Goat 7	Jugular	37	160	183	190	190	61	60
	Difference	64	79	61	53	50	40	50

¹ Point at which centrifuge has attained constant speed.

The marked hemoconcentration observed in these experiments is, therefore, consistent with the theoretical considerations, but the apparent tendency for hemoconcentration to continue for a short time *after* the stress has been terminated (see fig. 2) requires further explanation. As one possibility, we suggest that since the blood taken from the jugular vein was a mixed sample of the blood draining the entire head, it may be that the early samples represented blood from regions where stasis and deranged circulation had not been maximal. Later samples may include blood from regions where the stagnation and fluid loss had been more marked. If this suggestion is tenable, the time from termination of the run to peak concentration (minus the normal circulation time in the head) should be an index of the time required for the restoration of the local circulation. The time to peak concentration for both the hematocrit and plasma proteins should be about the same. These conditions are satisfied in the current experiments.

The fall of the curve, or hemodilution, may not be solely the result of the mixing of local blood with the general circulation. With the cessation of the stress, the intravascular hydrostatic pressure falls (table 2). This may leave a tissue hydrostatic pressure in excess of that within the capillaries so that water will move inward diluting both protein and erythrocytes.

Although the fall in concentration for these reasons should be, and was, quite rapid, the final equilibrium value cannot be predicted. A major factor in the determination of the final concentration of cells and proteins is the ratio between the volume of fluid lost and the total circulating blood volume. The fluid loss in turn is here dependent upon several factors. The difference between the intravascular and tissue pressure is determined by both the magnitude of g and the height of the effective hydrostatic column. The counterpressure of tissue fluids may rapidly become high in those regions bounded by water-restraining barriers (e.g., muscle sheaths, the cranium) while in looser areas (e.g., those of the face and neck) only minimal counterpressures will develop (21). In addition, the equilibrium level may possibly be modified by the drainage of the larger abdominal and thoracic lymphatic channels during the exposure to negative g . The relative importance of these factors must await further experimentation.

The discrepancy between the degree of hemoconcentration and plasma protein concentration was considered to be a qualitative indication of protein leakage. The occasional petechiae and frank hemorrhages observed in the stressed areas demonstrated that the force involved could cause vascular rupture. In non-hemorrhagic areas, the same stress might cause sufficient capillary distension to permit protein leakage while preventing, for the most part, the escape of red cells. The protein loss is probably due to mechanical rather than anoxic factors, since the stagnation is of relatively short duration. Capillary walls do not become permeable to protein as a result of anoxia unless the vascular oxygen saturation has been 15-25 per cent or less for 'several minutes' (22). On the other hand, Landis has shown that simple venous occlusion of the human arm at 80 mm. Hg leads to sufficient capillary distention to give 1.5 per cent protein in the filtrate (8). In these experiments, with the high venous and arterial pressures, the capillary pressure somewhere between these two will average at least twice 80 mm. Hg. The mechanical distention should thus be sufficient to allow great protein leakage and an average of 6.3 per cent protein in the filtrate is not an unreasonable figure. Significant protein concentrations in the filtrate from the circulation of men exposed to positive g have been reported (13).

In order to calculate the absolute volume of fluid accumulated in the stressed area, it would be necessary to know the rate of blood flow through that area. Nevertheless, the rapidity with which these changes of concentrations take place and their brief duration is striking. These experiments may be taken as further evidence of the ease and rapidity with which fluid may be transferred across the vascular wall under the influence of purely hydrostatic factors.

SUMMARY

1. The increased intravascular pressures developed during exposure to increased acceleration caused a rapid and significant outward filtration of fluid from the circulation.

2. Blood returning from the head end of goats after exposure to negative *g* showed a short, rapid concentrating phase, followed by a somewhat slower phase of dilution, with respect to both hematocrit and plasma protein concentrations.

3. At peak concentration there was evidence of significant leakage of protein from the circulation, as calculated from the hematocrit and plasma protein values.

4. Several mechanisms involved in the fluid loss and protein leakage are discussed.

We take this opportunity to acknowledge the interest and suggestions of Doctors D. R. Drury and C. F. Lombard in the formulation of the problem and the conduction of the experiments. Mr. T. M. Badgely rendered valuable technical assistance.

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SEQUENTIAL CHANGES IN OXYGEN CONSUMPTION DURING OLIGEMIC AND NORMOVOLIC SHOCK AND THEIR MEANING

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THE impression is widespread that oxygen consumption—and by inference metabolic rate—is decreased substantially in states of shock. A review of the limited number of studies made in indubitable states of shock makes it questionable whether the reduction in oxygen consumption noted is due to intrinsic disturbances of cellular metabolism or whether it is secondary to concomitant changes in the circulation and respiration.

A number of investigators, including Murlin and Greer (1), Gesell *et al.* (2), Schlomovitz and associates (3), Blalock (4) and Gollwitzer-Meier (5), have reported variable effects of bleeding on oxygen uptake. Among those who found reduction in oxygen consumption, no agreement appears to exist as to whether this is conditioned largely by the decline of blood pressure or by intrinsic changes in cellular metabolism. It is improbable that a state of shock existed in any of the experiments reported by the investigators mentioned.

Aub (6) reported a reduction in oxygen consumption in traumatized cats that were considered to be in states of shock when arterial pressure was reduced to 70 mm. Hg or less. The possibility that reduced oxygen uptake was occasioned by low arterial pressure was discussed but dismissed on rather meager evidence. Confirmatory results indicating that both oxygen consumption and basal metabolism are reduced in shock have been reported by Davis (7), Price (8), and Tabor and Rosenthal (9). In tabulations recently presented by Root, Walcott and Gregersen (10), a correspondence seems to exist between oxygen consumption and the degree to which cardiac output is reduced during states of traumatic shock.

In 1942 a method was devised in this laboratory whereby standardized hemorrhagic shock could be produced in dogs (11). This procedure has proved to be particularly suitable for a study of the dynamics of the heart and circulation. In order to round out the picture it was desirable to know whether irreversibility of the circulation thus induced is accompanied by reduced capacity of tissue cells generally to utilize oxygen. A study of this question was undertaken in this department by Green and Brofman in 1942, but this work was unfortunately interrupted. Their findings were reported briefly in a preliminary communication (12) and in greater detail in a master's thesis by Brofman (13). Dogs were bled in a graded manner. Usually 50 cc. of blood was withdrawn at 30-minute intervals until arterial pressure had declined to 40 or 50 mm. Hg. When this pressure was no longer maintained, it was supported by reinfusion of small quantities of blood until 25 per cent of the

Received for publication March 8, 1948.

hemorrhage volume had been returned. At this time the remaining volume of withdrawn blood was reinfused and circulatory failure followed rapidly. They found that oxygen consumption usually increased with each withdrawal of blood until mean arterial pressure fell to approximately 80 mm. Hg. At low pressure levels oxygen consumption decreased between 30 and 50 per cent. After reinfusion, oxygen uptake rose above control values until the arterial pressure again declined below 80 mm. Hg, after which it again decreased progressively. These investigators realized that changes in oxygen consumption are not wholly due to alterations in cellular metabolism but are complicated by the opposing effects of low arterial pressure and augmented respiration. In attempts to exclude everything except the cellular mechanisms, Green and Brofman devised elaborate and clever procedures in the hope that the effects of low arterial pressure and coincident dyspnea might be excluded. These attempts were not considered sufficiently successful to warrant statements regarding basic changes in cellular metabolism during shock. Consequently, they, perhaps wisely, refrained from published attempts to assess the relative interrelations between alterations in oxygen consumption and basal metabolic rate during shock.

In this investigation an effort was made to approach the problem from a different angle. It should be recalled that our procedure induces an irreversible circulatory state by maintaining a drastic posthemorrhagic hypotension for a stated interval of time, after which the blood volume is restored by reinjection of all the withdrawn blood. Various groups in this department have found that between 75 and 100 per cent of animals thus treated develop circulatory failure despite restoration of an essentially normal volume of blood. If the capacity of cells to utilize oxygen is basically damaged during the period of drastic hypotension during which circulatory irreversibility develops, it might be anticipated that such animals will continue to display a reduction in oxygen consumption after the circulating volume, cardiac output and arterial pressure have been restored to normal by reinfusion of blood.

METHODS

Dogs were anesthetized with morphine and sodium barbital, with sodium barbital alone, or with pentobarbital, all in biologically standardized doses. In other words, just sufficient amounts of these anesthetics were administered intravenously to produce a light but adequate and durable anesthesia. Oxygen consumption was recorded continuously by connecting the trachea with a recording Benedict-Roth respirometer. Mean arterial pressure was registered continuously from one femoral artery. Blood was withdrawn from the other femoral artery. Except in a few experiments in which the sciatic nerve was exposed and stimulated, no other operative procedures were carried out. Rectal temperature was maintained within a degree of the original temperature throughout the experiment by placing the dogs on a warmed animal board, and/or with the use of radiant lamps.

After a control period lasting from one to one and one half hours, standardized hemorrhagic shock was induced by bleeding according to the method developed in this laboratory (11). Briefly, this consisted in withdrawing blood at the rate of about 50 cc. per minute until mean arterial pressure had been lowered to 50 mm. Hg. This pressure level was maintained by withdrawing small additional quantities of blood. At the end of 90 minutes arterial pressure was further lowered to 30 mm. Hg by another small hemorrhage. This 30 mm. level was maintained for 45 minutes. At the end of that time, the withdrawn blood—properly heparinized, filtered, and warmed—was reinfused via a femoral vein and the redevelopment of circulatory failure awaited.

In previous analyses it was pointed out that while hemorrhagic shock begins

early in the hypotensive period an irreversible state does not eventuate until the middle of the 30 mm. Hg period. The successive stages were designated *oligemic shock*. The circulatory failure which develops after restoration of a normal blood volume was called *normovolemic shock* (14).

RESULTS

Effect of Oxygen Inhalation on Incidence of Irreversible Shock. Since these experiments differed from those in other series of animals in that the dogs were continuously breathing 100 per cent oxygen from the spirometer instead of atmospheric air, a comparison of the comparative incidence of irreversible shock seemed important. While this cannot be regarded as solving the disputed problem as to whether oxygen inhalation is beneficial in shock states, it does in our opinion have a bearing on the problem. Of the 12 dogs used in this investigation, two failed to develop shock. This 83 per cent incidence agrees with that found in several other series of experiments carried out by different teams. (For details see Wiggers, 15.)

Respiratory Changes and Oxygen Consumption. A description and analysis of typical effects requires graphic reproduction of four illustrative experiments shown in figure 1. These experiments are arranged according to the rapidity with which circulatory failure developed after reinfusion. Thus, the upper series of curves represent data from one of the two recovery dogs; the lower, from an animal that developed precipitous circulatory failure after reinfusion. Vertical lines demarcate the control period, the 50 mm. Hg posthemorrhagic hypotension, the 30 mm. period of hypotension and the postinfusion compensation followed by normovolemic shock. It may be mentioned that three of these experiments selected for illustration were carried out under sodium barbital anesthesia alone, and in one this anesthetic was preceded by a dose of morphine sulfate.

A survey of the plots from these typical experiments reveals that oxygen consumption is drastically reduced immediately after the decline of arterial pressure. The reductions ranged from 12.5 to 60 per cent in different experiments, and in nine of the most typical averaged 38 per cent. The posthemorrhagic reduction lasted for an hour or more, as indicated by A-B in figure 1. In some experiments (illustrated by *experiment 7*), the reduction occurred before respiratory minute volume had increased materially. In the majority of experiments, typified by *experiment 10*, the oxygen uptake remained low during this period despite considerable augmentation of pulmonary ventilation. Toward the end of the hypotensive period (B-C), and sometimes earlier, the rate of oxygen uptake increased. In fact, as shown in *experiments 8, 7 and 5* of figure 1, the oxygen consumption recovered practically to control levels when the end of the 50 mm. period was reached. In some of these experiments (e.g., *experiments 7 and 5*) this was accompanied by further accentuation of the pulmonary ventilation. However, the trends varied in different animals. The change in respiratory activity was much less abrupt or extensive in *experiment 8*. A material increase in oxygen uptake occurred from B to C in *experiment 10*, despite some reduction in pulmonary ventilation.

The second bleeding required to reduce arterial pressure to 30 mm. Hg levels at once reduced oxygen consumption significantly in all experiments (ranges 20 to 39

per cent below controls; average 30 per cent). This occurred even though the additional bleeding was never large; in some experiments the drop in arterial pressure

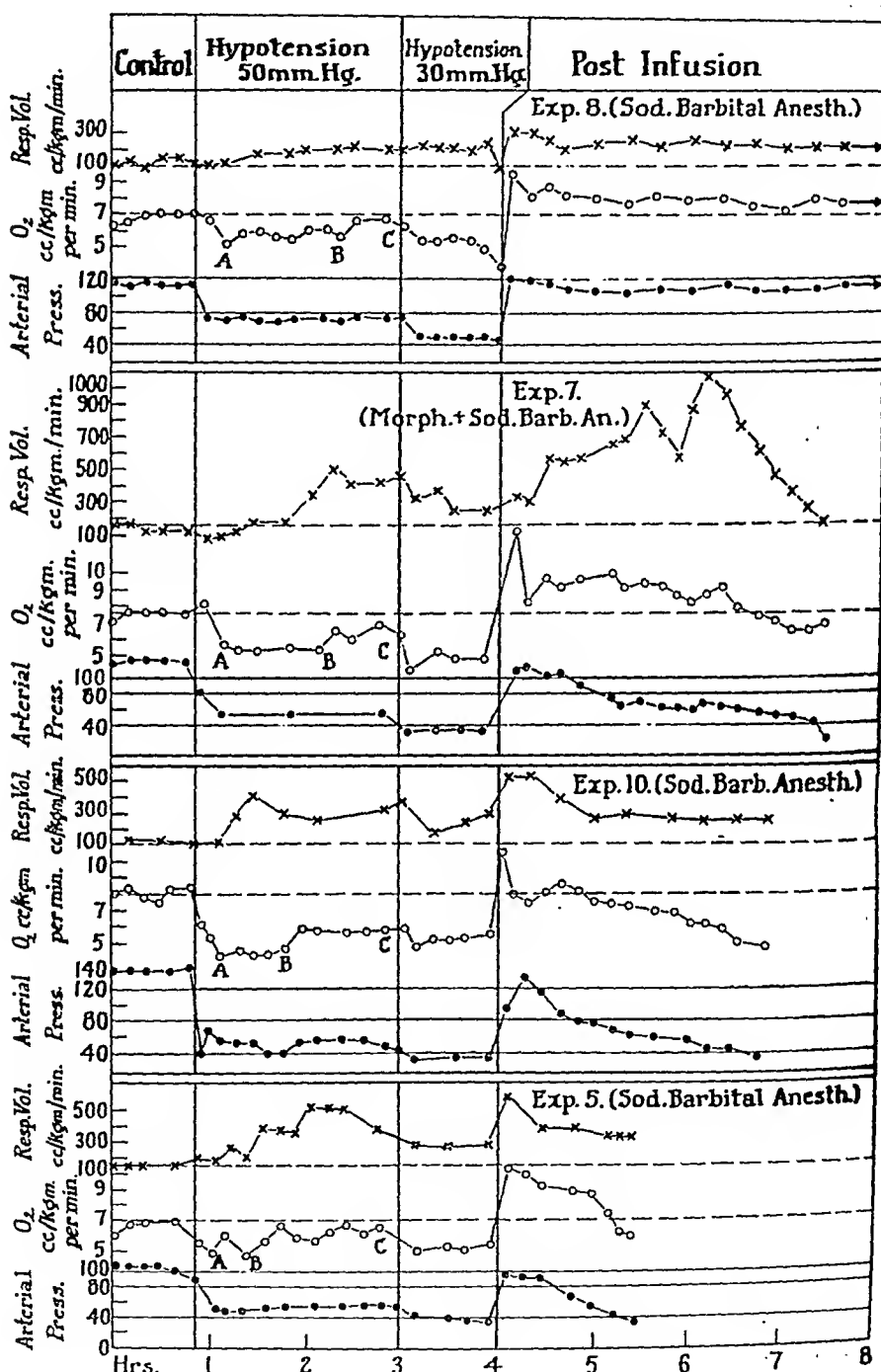


FIG. 1

supervened without additional hemorrhage. As indicated in the records of figure 1 some amelioration of the previous hyperpnea and pulmonary ventilation generally

developed during this period of 30 mm. hypotension (see *experiments 7, 10 and 5*). However, in a few cases, illustrated by *experiment 8*, oxygen consumption declined even when augmented respiratory efforts continued.

Reinfusion of the blood after irreversible circulatory changes had developed resulted in a pronounced increase over normal in the oxygen uptake (+30 to +85 per cent, average +61 per cent). This eventuated in spite of the fact that the blood which was reinjected intravenously was well oxygenated as a result of its exposure to the air. The large augmentation of oxygen consumption following reinfusion was not a temporary affair; usually it persisted for several hours, as illustrated in *experiments 8 and 7*; more rarely, it returned to a control level during the first hour following reinfusion, as in *experiment 10*. In many experiments, illustrated by *nos. 7 and 5*, the supernormal oxygen consumption even persisted after arterial pressure had begun its rapid decline during normovolemic shock. When this occurred it was generally attended by greatly augmented pulmonary ventilation. In most experiments oxygen uptake remained high even when arterial pressure had again been reduced to 50 mm. Hg. Reduction in oxygen consumption below control values such as are manifested in *experiment 10* was the exception rather than the rule.

INTERPRETATIONS AND SUPPLEMENTARY EXPERIMENTS

Since irreversible circulatory changes develop during the latter portion of the hypotensive period, the supernormal oxygen uptake which follows intravenous infusion of oxygenated blood and which is maintained even after circulatory failure recurs clearly indicates that reduction in oxygen uptake is not a necessary feature of the irreversibility picture.

Our results raise the question whether the smaller oxygen consumption which occurs during shock accompanied by significant hypotension (e.g., during periods of our experiments preceding infusion and in types of shock experiments reported by others) is in any way indicative of a real reduction in basal metabolic rate. The additional factors that must be evaluated before such a conclusion is reached will be briefly discussed as follows:

1. EXCESS OXYGEN CONSUMPTION INVOLVED AS A RESULT OF INCREASED RESPIRATORY EXERCISE. The increase in respiratory work which occurred during the course of our experiments is illustrated by changes in respiratory minute volumes plotted in figure 1. These curves reveal that as a rule respiratory activity is greatly augmented during all periods of oligemic and normovolemic shock. The use of morphine as a preanesthetic sometimes delays the supervention of hyperpnea, as in *experiment 7*, but certainly does not prevent its occurrence later.

The extent to which the increased respiratory effort can affect oxygen uptake in anesthetized dogs, such as ours, needed to be evaluated. This was done by inducing a similar dyspnea in four dogs by stimulation of a sciatic nerve. Data from two representative experiments are shown in figure 2. The marked elevation of mean arterial pressure and increase in pulmonary ventilation volume are obviously attended by a large increase in oxygen uptake. In *experiment 11* the oxygen uptake was doubled at the peak of respiratory activity. In *experiment 13* it increased 63 per cent over the control value.

The effect that augmented breathing during the posthemorrhagic hypotension

period has on oxygen consumption was evaluated as follows: after a second control period the animals were bled in the routine fashion. A 50 mm. Hg hypotension was maintained for approximately an hour, after which the augmented breathing was reduced or abolished by intravenous injection of morphine sulfate, 2 mgm. per kilo (fig. 2). It is obvious that the oxygen uptake decreased significantly as the respira-

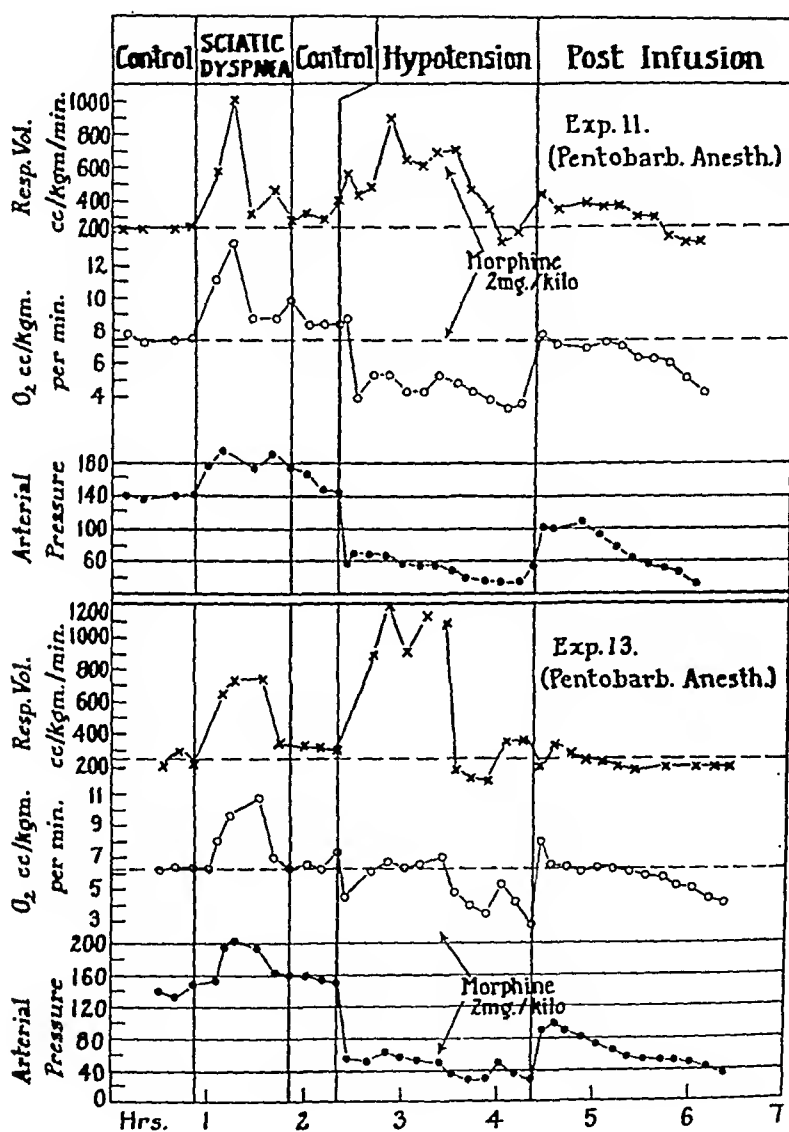


FIG. 2

tory minute volume again approached control levels. Obviously, any lowering of oxygen consumption which exists during such hypotensive periods as are exemplified by *experiment 11* does not depict the full reduction that can take place as a result of other forces. Similarly, when oxygen consumption recovers to control levels, as in *experiment 13*, this is clearly an effect of excessive breathing. It should be noted, however, that abolition of hyperpnea by morphine may have an additional indirect

action as well. As seen in both of these experiments, the arterial pressure falls with a reduction in respiratory activity. This has been attributed to removal of the pressor effect of respiration (16), which reduces venous return and cardiac output significantly under states of low arterial pressure. The possibility cannot be excluded that the reduced oxygen consumption following administration of morphine may be partly assigned to further impairment of oxygen transport capacity.

2. REDUCTION IN OXYGEN TRANSPORT CAPACITY. The decline in arterial pressure which supervenes after a large hemorrhage is due solely to reduction in cardiac output. Thereby the oxygen transport capacity becomes less, even though oxygen saturation of arterial blood may remain essentially unchanged. When this reduction reaches a critical level the excess oxygen required for augmented respiratory work can only be supplied immediately through a preferential increase in blood flow through muscles, or through a greater desaturation of its capillary blood. There is evidence in fact that the latter process starts to function before oxygen transport capacity has approached a critical level.

3. SURPLUS OXYGEN UPTAKE REQUIRED TO REOXYGENATE HIGHLY DESATURATED VENOUS BLOOD. It has been amply demonstrated that the oxygen content of mixed venous blood falls progressively during the development of shock. To this progressive decrease the return of highly deoxygenated blood from respiratory muscles must contribute considerably during the period of posthemorrhagic hypotension. Since the low cardiac output remains relatively stable during this period (17), the extra oxygen required to resaturate the blood fully must increase roughly at the same rate that the oxygen content of mixed venous blood decreases. This probably accounts for a part of the increased oxygen uptake during the periods of hyperpnea. Through this reserve mechanism the overactive respiratory muscles are able to borrow additional oxygen from the blood during periods when the oxygen transport capacity is strained. At the same time, the increased pulmonary ventilation provides the means by which the extra oxygen used may be replenished during the period of a single circulation.

4. SURPLUS OXYGEN REQUIRED FOR OXIDATION OF ACCUMULATED PRODUCTS OF ANAEROBIC METABOLISM. *Repayment of oxygen debt.* The development of a metabolic type of acidosis during states of prolonged hypotension offers good evidence that the tissues of the body, including the respiratory muscles, revert partly at least to a type of anaerobic metabolism. When the oxygen transport capacity is increased as a result of a large blood transfusion the oxidation of accumulated products of anaerobic metabolism obviously requires an extra uptake of oxygen. Thus, together with persistence of augmented breathing, probably accounts for the continued large oxygen uptake after reinfusion of the withdrawn blood.

In order to substantiate this plausible interpretation, the augmented respiratory activity which persists after reinfusion was abolished by administering a dose of morphine. Two typical experiments are shown in figure 3. In *experiment 14* morphine produced an unusual depression of respiratory activity. Nevertheless, oxygen consumption was not greatly decreased. In *experiment 15* the respiratory minute volume was restored exactly to control values for several hours. During this period the oxygen consumption remained significantly elevated.

5. IMPAIRMENT OF OXYGEN UTILIZATION BY TISSUES GENERALLY. The progressive development of a metabolic acidosis during prolonged posthemorrhagic hypotension is also evidence that the basal metabolic needs for oxygen were not satisfied. Consequently, the existence of a state of decreased oxygen utilization cannot be denied, even when it is obscured by operation of other factors. The important question under discussion is whether a permanent and perhaps irreversible metabolic disturbance has been created or whether the reduction in oxygen utilization

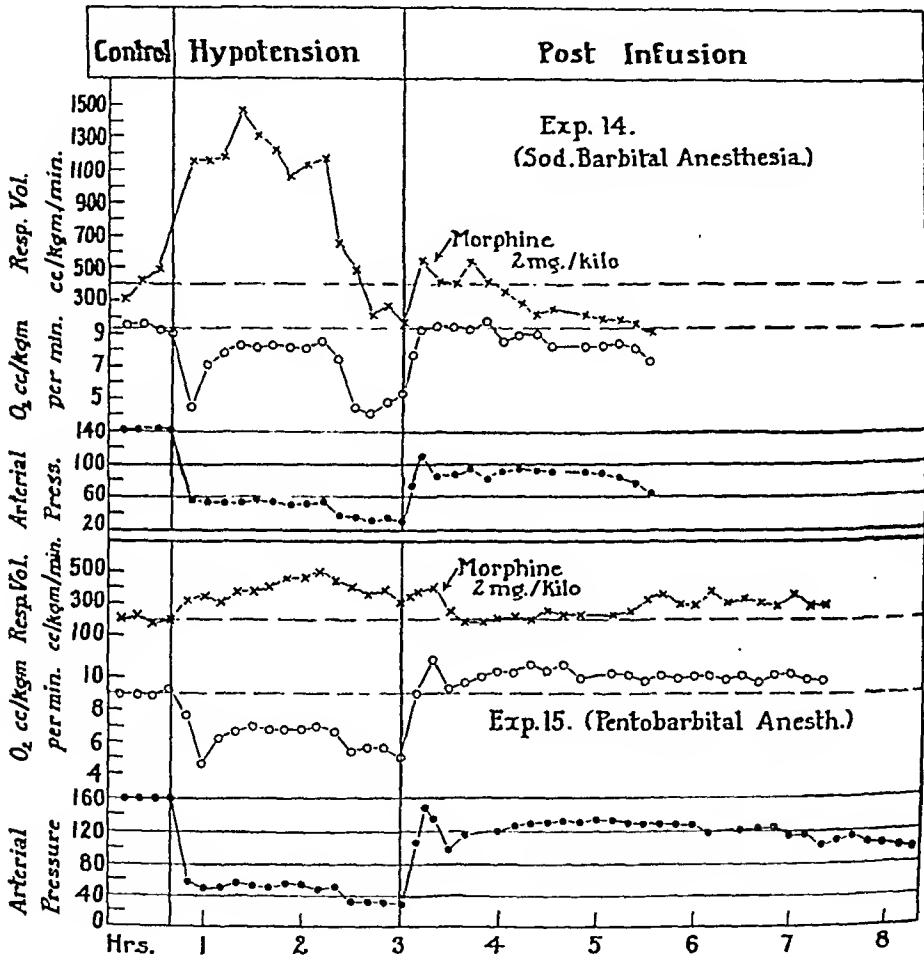


FIG. 3

is merely secondary to impairment of blood flow, reduction in oxygen transport capacity and, in certain cases, to lowering of body temperature. When shock is induced by other procedures a decline in body temperature usually occurs. This unquestionably causes a reduction in basal metabolic rate, as measured either by oxygen uptake or by direct calorimetry. It is by no means demonstrated, however, whether cooling acts by deceleration of chemical reactions generally or through depression of respiratory, cardiac and other visceromotor activities. Correlations of findings with Van't Hoff's law may perhaps result through changes of a physiological rather than a physiochemical nature. However, changes in velocity of chemical

reactions due primarily to a decline in body temperature was minimized in our experiments by our practice of maintaining rectal temperatures within 1°C. of that at the start of an experiment. The appendages which do cool could not affect oxygen uptake materially. Their metabolizing tissues constitute only a small fraction of the body weight and, moreover, blood flow to the hind legs was materially reduced since both femoral arteries were ligated.

The changes in oxygen utilization due to intrinsic cellular damage are difficult to assess, because so many factors combine to elevate oxygen uptake after normal blood volumes, normal cardiac output and normal arterial pressures have been restored through blood transfusion. Together they could easily obscure any persistent depression in oxygen utilization due to cellular injury. However, if our premises are correct, that the tremendous increase in oxygen consumption after transfusion is partly referable to oxidation of metabolic acids, the inference appears allowable that the body cells concerned with their oxidation have not been damaged irreversibly.

Explanation of Changes in Oxygen Consumption During Successive Phases of Oligemic and Normovolemic Shock. We have discussed several determinants which in addition to reduced oxygen utilization by cells may affect the oxygen consumption. The balance of these factors undoubtedly varies considerably at different periods of oligemic and normovolemic shock in an individual experiment and during similar periods in different animals. The dominance of one or the other factor can, however, generally be recognized. It is highly improbable, for example, that a generalized decline in basal metabolic rate develops immediately after a large loss of blood. Nevertheless, oxygen uptake decreases immediately and continues for some time. This reduction is more likely due to the predominant effect of lowered cardiac output and oxygen transport capacity. The progressive and in some cases complete recovery of oxygen uptake which ensues during the latter portion of the 50 mm. Hg period can be accounted for by the excess oxygen required for augmented respiratory activity.

The mechanisms by which more oxygen can be made immediately available to respiratory muscle without any necessary improvement in oxygen transport capacity has been analyzed above. The sharp decrease in oxygen consumption which ensues during the 30-mm. period is attributed in part to further reduction in oxygen transport capacity by a second bleeding and partly to diminution in amplitude and rate of respiration. When respiratory depression actually occurs, reoxygenation of blood becomes less complete, the heart slows rapidly, arterial pressure declines and respiration ceases unless prompt resuscitative measures are taken. It is highly questionable whether the lowered oxygen uptake during this critical period in any way reflects a generalized reduction in oxygen consumption due to cellular damage; the reduction is certainly dominated by cardiac and respiratory responses and their mechanical effects on oxygen uptake. Incidentally, it is important to note that breathing 100 per cent oxygen cannot avert a crisis, whereas small infusions of whole blood which raise the cardiac output and oxygen transport above liminal values are effective. The large increase in oxygen consumption which follows a transfusion and continues even as circulatory failure redevelops is attributed to the combination of

three factors, namely, improvement in oxygen transport, continuance of augmented respiratory action plus oxidation of metabolic acids.

SUMMARY

The oxygen consumption of barbitalized dogs was recorded continuously during progressive oligemic and normovolemic shock. This was produced by massive hemorrhage and subsequent reinfusion of the withdrawn heparinized blood, according to standardized procedures developed in this laboratory. An analysis of the results supports the following conclusions:

1. Reduction in oxygen consumption is not a necessary concomitant of the irreversibility picture.

2. Even when body temperature is kept constant oxygen consumption is not a measure of basal metabolic rate during shock. This is due to the fact that oxygen uptake is predominately affected by reduction in oxygen transport capacity, excessive respiratory effort and varying degrees of acidosis with accumulation of oxygen debt.

3. Neither these experiments nor those published by others offer clear-cut evidence for the generally accepted hypothesis that development of an irreversible circulatory state is accompanied by overall cellular damage which manifests itself by reduced oxygen utilization.

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EFFECT OF PENTOTHAL SODIUM ON BLOOD GAS TRANSPORT^{1, 2}

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DURING the course of some experiments involving blood gas studies consistently low arterial O₂ contents of our dogs led to a suspicion of some effect of the anesthetic *per se*. Accordingly comparison of pre- and post-anesthetic blood gas contents indicated an appreciable difference, the cause for which was not readily apparent.

At the time these experiments were begun no reference was found in the literature relative to the effect of pentothal on blood gases. Reference has been made many times to the hemodilution effect of barbiturates in general, however, as will be discussed later. Many authors have referred to a depression, or arrest, of respiration following pentothal; in fact Draper and Whitehead (1) used it to produce respiratory arrest in dogs. However, since our animals did not show a grossly apparent lessening of respiration after the anesthetic, we did not believe this could wholly account for our results. Hence it seemed desirable to investigate the mechanism whereby pentothal reduced the oxygen content of the blood.

METHOD

Mongrel dogs were used. Under local anesthetic (procaine³, 2 per cent), blood was drawn from the femoral artery and vein (or in some cases just the artery) into heparinized syringes and immediately refrigerated either in the sealed off syringes or after transfer to mercury-filled storage tubes. Five to 10 cc. of freshly dissolved 5 per cent pentothal sodium were then administered into the femoral vein. Approximately one milliliter beyond that required to elicit the customary sigh was found to ensure deep narcosis.

After a five-minute wait another pair of blood samples was drawn and handled as before. All samples were analyzed for O₂ and CO₂ content by the usual Van Slyke manometric techniques within four hours of being drawn.

In a second series of experiments the above procedure was followed, except that as quickly as possible after the pentothal injection a tracheal cannula or tube was inserted and artificial respiration with room air applied. Hematocrit studies were made on this and succeeding series.

The third and fourth series of experiments were repetitions of series I and II,

Received for publication March 8, 1948.

¹ Presented in part at the Federation Meetings, Chicago, Illinois, 1947.

² Work done under Contract No. W33-038 ac 14757 with the Aero Medical Laboratory, Air Materiel Command, U.S.A.F., Wright Field, Ohio.

³ Kindly donated by Bristol Laboratories.

respectively, except that following the withdrawal of the post-pentothal blood sample, 5 to 8 cc. of 1:10,000 epinephrine were injected I.V. After a five-minute wait the third set of blood samples was taken.

TABLE 1. OXYGEN, CARBON DIOXIDE AND HEMATOCRIT VALUES OF ARTERIAL BLOOD OF DOGS BEFORE ANESTHESIA, AFTER PENTOTHAL NA AND AFTER EPINEPHRINE FOLLOWING PENTOTHAL *Series I* and *III* were with spontaneous respiration: *series II* and *IV*, with artificial respiration following pentothal administration

SE- RIES	NO. DOGS		OXYGEN			HEMATOCRIT ¹			CARBON DIOXIDE		
			Volume %	Difference		cc./100 cc.	Difference		Vol. %	Difference	
				Vol. %	% Norm.		Absol.	% Norm.		Vol. %	% Norm.
I	12	Before pento- thal	18.7 ±2.06 ²						36.8 ±7.12		
		After pentothal	15.6 ±2.52	-3.1	-16.6				43.1 ±5.28	+6.3	+17.1
II	10	Before pento- thal	18.2 ±2.26			43.2 ±4.53			41.5 ±3.03		
		After pentothal	16.5 ±2.15	-1.7	-9.4	38.9 ±4.00	-4.3	-10.0	37.8 ±6.07	-3.7	-8.9
III	7	Before pento- thal	18.8 ±1.49			41.1 ±4.21			41.7 ±3.60		
		After pentothal	14.3 ±1.31	-4.5	-23.0	38.0 ±4.45	-3.1	-7.5	48.7 ±4.67	+7.0	+16.8
		After epineph- rine	20.2 ±2.86	+1.4	+7.4	46.2 ±6.39	+5.1	+12.4	42.9 ±3.73	+1.2	+2.9
IV	6	Before pento- thal	18.5 ±2.12			42.1 ±3.28			40.8 ±3.22		
		After pentothal	17.2 ±2.05	-1.3	-7.0	38.1 ±3.60	-4.0	-9.5	34.6 ±5.17	-6.2	-15.2
		After epineph- rine	20.3 ±2.35	+1.8	+9.7	43.3 ±4.32	+1.2	+2.8	31.1 ±3.92	-9.7	-23.8

¹ Winthrobe tubes. ² Standard deviation.

RESULTS

The mean and standard deviation of the arterial O₂ and CO₂ contents and hematocrit values are listed in table 1. Also listed in this table are the differences, in per cent of the control values, for each of the three means following both pentothal and, where used, epinephrine administration. To facilitate evaluation of the effect

TABLE 2

Analysis of data from table 1. The algebraic sums and differences of the changes observed in table 1 are shown and apparent causes ascribed

SERIES NO.	PROCEDURE			O ₂ CHANGE % norm.	HEMATOCR. CHANGE % norm.	CO ₂ CHANGE % norm.	REMARKS
	Pento- thal	Arti- ficial resp.	Epi- nephrine				
I	+	o	o	-16.6		+17.1	
II	+	+	o	-9.4	-10.0	-8.9	Reversal of CO ₂ sign due to hyperventilation
				-7.2			Difference due to respiratory depression
						-26.0	Total CO ₂ change due to artificial respiration
III(a)	+	o	o	-23.0	-7.5	+16.8	
III(b)	+	o	+	+7.4	+12.6	+2.9	
				+30.4	+20.1	-13.9	Effect of epinephrine (spontaneous respiration) O ₂ increase in excess of hematocrit change, and CO ₂ decrease represent epinephrine stimulation of circulatory and/or respiratory mechanisms on gas exchange
IV(a)	+	+	o	-7.0	-9.5	-15.2	CO ₂ sign shows hyperventilation
IV(b)	+	+	+	+9.7	+2.8	-23.8	" " " " and epinephrine effect
				+16.7	+12.3	-8.6	Magnitude of epinephrine effect in presence of artificial respiration
III(a)	+	o	o	-23.0	-7.5	+16.8	Respiratory and hematocrit depression due to pentothal
IV(a)	+	+	o	-7.0	-9.5	-15.2	
				+16.0	-2.0	-32.0	Magnitude of effect of artificial (hyper) ventilation on pentothal depression
III(b)	+	o	+	+7.4	+12.6	+2.9	
IV(b)	+	+	+	+9.7	+2.8	-23.8	
				+2.3	-9.8	-26.7	Effect of artificial respiration in presence of epinephrine

of the several procedures used a second tabulation of these differences, arranged for ready comparison, is made in table 2.

It is apparent from the figures of series I that pentothal causes a considerable reduction in the arterial O_2 content and a comparable percentage increase in CO_2 . However, reasoning on the basis of the shapes of the O_2 and CO_2 dissociation curves suggests that respiratory depression alone could not account for the observed fall in O_2 . Accordingly, in series II, hyperventilation was artificially induced during the period of anesthesia. The effect of respiratory depression was thus removed as evidenced by the fall in CO_2 content to below the preanesthetic level. The fall in O_2 with pentothal was then nearly halved, but not obliterated. Hematocrit studies in this series showed that the remainder of the fall in O_2 following pentothal could be accounted for by the reduction in the volume of red cells per unit volume of circulating blood.

The reduction in hematocrit should be relieved by the injection of epinephrine. In series III the dogs were again allowed to breathe spontaneously throughout the experiment. The pentothal in this series caused a somewhat greater fall in O_2 than in series I, but epinephrine injected during this depression was able to raise the O_2 content to a level even slightly above the control value. The CO_2 excess was reduced but not entirely overcome and the hematocrit was raised considerably above the control value.

In series IV, in the presence of artificial respiration, pentothal again reduced the O_2 content and the hematocrit value to approximately equivalent degrees and the epinephrine again overcompensated the effect of the pentothal and further reduced the already lowered CO_2 content.

The effect of artificial respiration *per se* on arterial O_2 , CO_2 and hematocrit is apparent by comparing the postpentothal levels in series III(a) and IV(a). With controlled respiration the O_2 content fall is 16 per cent less, the hematocrit decrease is greater by two per cent, and the CO_2 is reduced from +16.8 per cent to -15.2 per cent. That is to say, artificial ventilation can restore the normal arterial O_2 and CO_2 tensions but cannot restore the O_2 carrying capacity.

Epinephrine injections on the other hand, with or without artificial respiration, (compare series III(a) and (b) and IV (a) and (b)) may restore the O_2 capacity to a level above the normal and the O_2 and CO_2 tensions to levels near the normal. It is thus apparent that epinephrine can reverse the pentothal-induced changes in blood gases about as effectively as artificial ventilation, both by restoring the hematocrit and by enhancing gaseous exchange through stimulation of circulatory and/or respiratory mechanisms.

It is noteworthy that in the presence of marked hypoxia following pentothal (as obtains during spontaneous respiration) the hematocrit decrease is not as great as occurs during artificial ventilation. Hypoxia as such prevents the full pentothal effect on the hematocrit. To this extent epinephrine and hypoxia are synergistic, if in fact the mechanisms are not identical.

Although the respiratory depression and hematocrit decrease appear to account fully for the observed fall in arterial O_2 content, O_2 capacity studies were carried out on 10 dogs to investigate a possible reduced affinity of hemoglobin for O_2 . The results were negative.

DISCUSSION

While this work was in progress a paper by Barton, Wicks and Livingstone (2) appeared in which observations were presented on the arterial O_2 content of six humans and six dogs following intermittent fractional dose administration of pentothal. The total time of administration was 10 to 44 minutes in dogs and blood samples were drawn 10 minutes after completion of the anesthetic. Five of these dogs showed a fall in arterial O_2 content and one a very slight rise. The average fall for the six dogs was 2.32 vol. per cent, or 10.5 per cent. Our findings are in essential agreement with theirs since their animals were in an intermediate respiratory state between our two categories of considerable depression via deep narcosis and no depression via artificial respiration during narcosis. Our overall figures for arterial O_2 content reductions, (combining series I and III) averaged -19.2 per cent for the animals allowed to breathe spontaneously and -9.3 per cent for those animals receiving artificial respiration.

From these figures it would appear that the fall in O_2 content of the blood following pentothal administration is about equally due to respiratory depression and to hemodilution. The two are somewhat interrelated, however, inasmuch as the hypoxia resulting from the reduced respiration may tend to oppose the hemodilution. Evidence for this is found in table 1 in the comparison of the degree of hematocrit fall in series II or IV (-10.0 and -9.5 per cent,) where the respiratory level was maintained artificially and the O_2 contents were below the control values by only 9.4 and 7 per cent, respectively.

The effect of barbiturates in general on the blood picture has been referred to many times. Bourne, Bruger and Dreyer (3) noted the hydremic effect of sodium amytal without offering an explanation. Searles and Essex (4) reported that splenectomy abolished the dilution effect of Na amytal, and Hausner, Essex and Mann (5) showed by X-ray visualization of the spleen that that organ is markedly enlarged in dogs following amytal, nembutal or pentothal. However Adolph and Gerbasi (6) Bollman, Svirebely and Mann (7) and Jarcho (8) showed that splenic abstraction only partially accounts for the hemodilution following barbiturates and an actual increase in circulating plasma ensues. Hamlin and Gregerson (9), through blood volume studies, and Polderman, McCarrell and Beecher (10), through studies of lymph flow, both concluded that barbiturates tend to withdraw fluid from tissue spaces into the blood stream.

In the anesthetized animal epinephrine would be expected to oppose the hemodilution effects caused by both red cell storage and plasma increase (9, 11). Our results on postpentothal epinephrine injections are interesting in that not only do they oppose the dilution effect but also enhance the respiratory exchange both in those animals breathing spontaneously, as shown by a reduction in the CO_2 excess, and in those receiving artificial respiration, as shown by a further reduction in the already lowered CO_2 .

CONCLUSIONS

1. Pentothal sodium injection to the stage of deep narcosis lowers the O_2 content of dogs by some 19 per cent. By preventing respiratory depression via artificial respiration the fall in O_2 content is only about 9 per cent, all of which can be ac-

counted for by a decreased hematocrit. It thus appears that the total fall in arterial O_2 content observed following pentothal injection is approximately equally chargeable to respiratory depression and to hemodilution.

2. The affinity of the hemoglobin for oxygen is not interfered with as evidenced by O_2 capacities.

3. The hemodilution effects, and to a considerable extent the respiratory depression, can be effectively opposed by the injection of epinephrine.

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SUSCEPTIBILITY OF CATS AND DOGS TO PROGRESSIVE ANOXIA

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IT HAS been known for many years that various species of animals differ widely in their susceptibility to anoxia. Paul Bert (1) in his book, *La Pression Barometrique*, the cornerstone of modern aviation medicine, gives considerable evidence that cats are more susceptible to oxygen lack than are dogs. Campbell (2) reported experiments which indicated that cats do not acclimatize to oxygen lack as well as other species. Dill (3) also called attention to this difference between cats and other species.

A knowledge of the physiological basis for these differences would throw light on the fundamental mechanism involved in the supply of oxygen to the tissues and would probably be of value in the development of methods for the protection of man against anoxia. A comparative study of the responses of cats and dogs to anoxic anoxia has therefore been made.

METHODS

Experiments were performed on 27 dogs and 17 cats. All animals were adults and both males and females were used. The animals were anesthetized with nembutal (30 mgm. per kgm., intraperitoneally) except for two cats that received urethane (1.25 grams per kgm., intraperitoneally).

Anoxia was produced by connecting the respiratory tract of the animals to a rebreather apparatus consisting of a spirometer in a closed circuit containing a soda lime cannister for the absorption of carbon dioxide. The size of the spirometer and the initial oxygen concentration were chosen so that respiratory failure occurred in about ten minutes. Respiratory rate and volume were recorded from the rise and fall of the spirometer bell. Blood pressure was recorded with a mercury manometer from the right femoral artery. In general, the procedure was as follows. After the necessary surgery had been completed but while the animal was still breathing room air, a sample of arterial blood was drawn from the left femoral artery with anaerobic precautions. The animal was then switched to the spirometer circuit and recording begun. Recording was continued until the respiratory movements ceased. A second sample of arterial blood was taken at this instant. The animal was then switched to room air and resuscitated. A sample of the air left in the spirometer was analyzed for oxygen and carbon dioxide content. After a recovery period of from 20 to 30 minutes the chemoreceptors were denervated by dissection and phenolization of both carotid sinus regions and bilateral vagotomy.

A period of 15 to 20 minutes was allowed for recovery from these surgical procedures and for stabilization of the blood pressure. The experiment was then repeated.

Blood samples were analyzed for oxygen and carbon dioxide content by the Van Slyke manometric method, and the oxygen capacity of one of the samples was determined. The air samples were analyzed for oxygen and carbon dioxide content with the Haldane apparatus.

Measurements of respiratory rate and depth, heart rate and arterial blood pressure were made

Received for publication April 12, 1948.

during the first and last minutes of each experiment. Changes in these values are expressed in terms of percentage deviation from the initial values.

RESULTS

A. Response of intact animals. The data on the composition of inspired air at the moment of respiratory failure and on the O₂ and CO₂ content of arterial blood are given in table 1. These data show conclusively that respiratory failure occurred in the cat when the percentage of oxygen in the inspired air was much higher than the corresponding values tolerated by the dog. Thus, on the average, the dog was able to continue breathing until the O₂ content of the inspired air reached 2.62 per cent while the cat ceased breathing at 4.66 per cent oxygen. Determinations of oxygen saturation of arterial bloods at the moment of cessation of respiration reveal minor differences between the two species.

TABLE 1. INSPIRED AIR OXYGEN AND BLOOD GASES AT THE MOMENT OF CESSATION OF RESPIRATION

		Inspired air oxygen content	Arterial oxygen saturation	Arterial CO ₂ content
Intact dog	mean	2.62%	11.6%	35.9 vpc
	no. expts	26	9	9
	range	1.60-4.00	8.2-22.1	25.8-43.7
Intact cat	mean	4.66%	7.8%	34.5 vpc
	no. expts	15	6	6
	range	2.96-7.84	2.6-22.1	30.7-40.1
Denervated dog	mean	7.00%	10.1%	40.2 vpc
	no. expts	13	9	9
	range	1.72-15.8	4.6-11.6	31.0-52.8
Denervated cat	mean	7.96%	7.4%	38.0 vpc
	no. expts	8	6	6
	range	4.81-10.8	3.7-13.8	23.6-45.2

Comparison of the respiratory responses to reduced O₂ tension of the two species (table 2) reveals a marked difference. The dog responded to anoxia with an average increase in ventilation volume of 370 per cent while the cat showed an average increase of only 54 per cent. In both species, the rate of respiration showed a greater increase than did the depth.

As might be expected, the average carbon dioxide content of arterial blood, shown in table 1, tends to decrease with increasing ventilation volume.

The circulatory responses of both dogs and cats are given in table 3. The dog showed an average increase in heart rate of 11.8 per cent and an increase in mean arterial pressure of 11 per cent. The cat, on the other hand, showed an average decrease of 13 per cent in mean arterial pressure and an average decrease of 14.9 per cent in heart rate.

B. Responses of the denervated animals. Denervation of the chemoreceptors

practically obliterated the species differences noted in intact animals and reduced the anoxic tolerance of both dogs and cats. The denervated dog stopped breathing when the oxygen content of inspired air was reduced to 7.00 per cent, while respiratory failure in the denervated cat occurred with the oxygen content of inspired air reaching

TABLE 2. RESPIRATORY RESPONSES TO PROGRESSIVE ANOXIA

		Respiratory rate	Tidal volume	Ventilation volume
				cc/kgm/min.
Intact dog	mean	+196%	+46%	+370%
	no. expts	28	28	28
	range	+400%—+77%	+102%—(—12%)	+668%—+152%
Intact cat	mean	+27%	+18%	+54%
	no. expts	17	17	17
	range	+33%—(—25%)	+67%—(—9%)	+120%—0%
Denervated dog	mean	+56%	+6%	+24%
	no. expts	13	13	13
	range	+120%—(—50%)	+119%—(—53%)	+129%—(—18%)
Denervated cat	mean	+11%	+13%	+12%
	no. expts	8	8	8
	range	+93%—(—12%)	+50%—0%	+190%—0%

TABLE 3. CIRCULATORY RESPONSES TO PROGRESSIVE ANOXIA

		Arterial pressure	Heart rate
Intact dog	Mean	+11%	+12%
	no. expts	27	9
	range	+33%—+2%	+29%—(—6%)
Intact cat	Mean	—13%	—15%
	no. expts	15	6
	range	+9%—(—32%)	—5%—(—33%)
Denervated dog	Mean	—23%	+7%
	no. expts	13	9
	range	+27%—(—50%)	+16%—(—6%)
Denervated cat	Mean	—24%	+7%
	no. expts	8	6
	range	—2%—(—41%)	+18%—(—5%)

7.96 per cent. The values for arterial oxygen did not change significantly from those for the intact animals (table 1).

The increase in ventilation volume of the denervated cat and dog was 12 per cent and 24 per cent, respectively (table 2), and the arterial carbon dioxide content of both animals was correspondingly higher than those of the intact animals (table 1).

With denervated animals the circulatory responses to anoxia were the same in both species (table 3). Arterial pressure dropped 23 per cent in the dog and 24 per cent in the cat, while the heart rate increased 6.5 per cent in the dog and 6.7 per cent in the cat. The responses of the cats anesthetized with urethane did not differ from those anesthetized with nembutal.

DISCUSSION

These data definitely prove that under the conditions of these experiments, there is a difference in susceptibility of cats and dogs to anoxia. The outstanding difference lies in the respiratory response, the dog showing about six times the hyperpnea of the cat. When this hyperpnea is prevented by chemoreceptor denervation the species difference disappears. It appears, therefore, that there is little or no difference in the susceptibility of the cells of the medullary respiratory center of these two species to progressive anoxia. This conclusion is confirmed by the data on arterial pO_2 at the moment of respiratory failure (see table 3).

The greater ability of the dog to withstand anoxia depends primarily on his ability to decrease the gradient between inspired pO_2 and alveolar pO_2 by hyperventilation, and thus to maintain a more adequate alveolar pO_2 in the face of a decreasing O_2 content of inspired air. This maintenance of alveolar pO_2 is brought about partly by a reduction of the alveolar CO_2 as indicated by the drop in arterial CO_2 values, but to a greater extent by increased mixing of alveolar and inspired airs as a result of hyperventilation.

Figure 1 shows the relation of ventilation volume to the percentage of O_2 in inspired air at the moment of respiratory failure (alveolar $pO_2 = 10$ mm.).

It is evident that as ventilation increases, the pO_2 of alveolar air approaches that of the inspired air. Theoretically, therefore, with infinite ventilation the ratio of inspired air pO_2 to alveolar air pO_2 would be 1.0. On the other hand, if there were no ventilation the alveolar pO_2 would tend to approach zero and the above ratio would therefore approach infinity. This relationship may be expressed mathematically by the equation $R = \frac{A}{\text{Vent. Vol.}} + 1.0$, where A is a constant expressing the relation of ventilation volume to mixing efficiency. Factors which effect the value of the constant A are changes in the alveolar CO_2 , effects of depth and rate of breathing, the vital capacity, the volume of the dead space and perhaps other less obvious factors.

When inspired air pO_2 /alveolar pO_2 ratios are calculated from the data of these experiments (assuming an alveolar pO_2 of 10 mm. Hg at the moment of respiratory failure) and plotted against ventilation at that instant, the experimental curve approaches the theoretical (see fig. 1). As might be expected, the curve indicates that as ventilation volume increases, the benefit derived from this increase becomes progressively less. Thus the advantage held by the dog over the cat in ability to maintain alveolar pO_2 is gained at the expense of a markedly disproportionate increase in ventilation volume.

It is of interest that these results are essentially similar to those reported by Houston (4) in a study of the effects of hyperventilation in humans exposed to low oxygen mixtures.

While the primary cause of the species difference here reported seems to lie in the respiratory adjustments, it is well to point out that circulatory responses paralleled those of respiration in both species. The lack of cardiovascular stimulation by anoxia in the case of our chemoreceptor intact cats is at variance with the usually reported data. It is our opinion that this difference may be dependent upon the rate of induction of anoxia, an hypothesis which is now being experimentally tested.

Finally, the fact that these experiments have been performed under barbiturate anesthesia must be taken into account in interpretation of the findings.

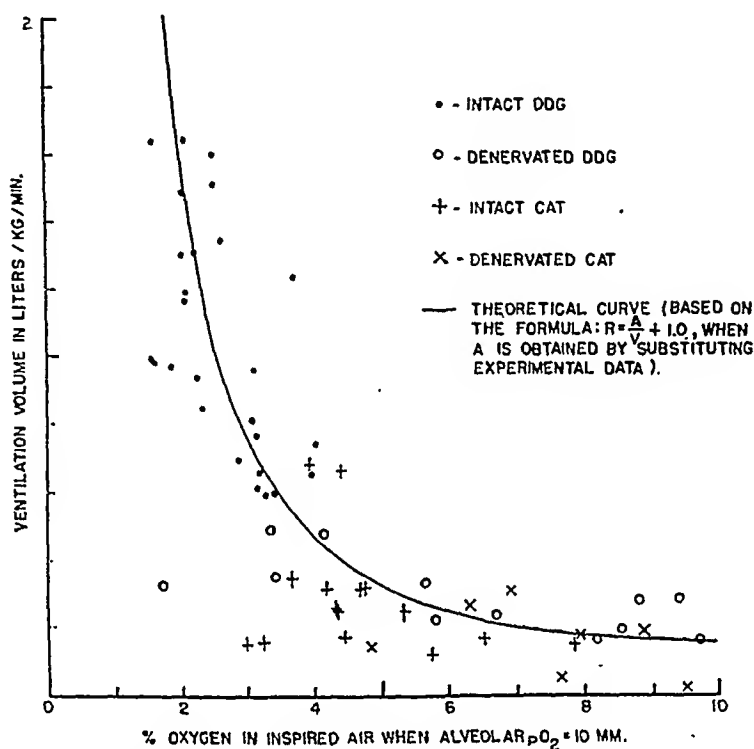


Fig. 1. COMPARISON OF EXPERIMENTAL DATA with values obtained from theoretical considerations of the relation between ventilation volume and alveolar pO_2 at the moment of respiratory failure.

SUMMARY

A study was made of the respiratory and circulatory adjustments to progressive anoxia of the cat and dog.

The cells of the medullary respiratory center of the cat and dog show only minor differences in the level of arterial O_2 saturation at which they cease to function. However, the cat stops breathing at a much higher inspired air pO_2 than does the dog.

In the absence of the chemoreceptors, both species are less resistant to and show similar respiratory responses to anoxia. With intact chemoreceptors the dog hyperventilates to a much greater degree than does the cat. The effect of this hyperventilation on the alveolar pO_2 is the primary factor concerned in the species difference.

A species difference in circulatory responses to anoxia was also demonstrated.

In the intact dog, arterial pressure increases 11 per cent, while in the intact cat it decreases 13 per cent. When the chemoreceptors are denervated, the arterial pressure of both cat and dog decreases about 24 per cent in response to progressive anoxia.

A theoretical expression of the relation between ventilation volume and the inspired air pO_2 /alveolar air pO_2 ratio is presented.

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METABOLIC CHANGES OF THE RESTING POTENTIAL IN RELATION TO THE ACTION OF CARBON DIOXIDE^{1, 2}

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IN SYSTEMS like frog skin (1) and frog nerve (2) the comparatively steady differences of potential, generally referred to as resting potentials, are supported by respiration under aerobic conditions and, to some extent, by glycolysis during anaerobiosis. Such potentials are good current generators (3), even under conditions of depressed metabolism (4). Furthermore, they vary linearly with the logarithm of the extracellular potassium concentration although potassium inhibits (5), or first accelerates and then inhibits (6), respiration over a more limited range of concentration.

Such observations would be explained if the primary source of potential were the potassium gradient across the cell membranes and if the function of metabolism simply consisted of maintaining this gradient. The metabolic processes could contribute to the gradient by producing hydrogen ions for exchange with extracellular potassium, CO₂ and lactic acid serving as sources of hydrogen ions under aerobic and anaerobic conditions respectively.

The experiments to be described were designed to test for the operation of this mechanism in frog nerve. Anoxia and the subsequent return to oxygen were selected as a means for modifying CO₂ production by the fibers. Since carbonic anhydrase in the fibers might be catalyzing hydrogen ion formation from CO₂ or H⁺ disappearance, the action of inhibitors of this enzyme on the potential changes under these conditions was studied. The effects of 5 per cent CO₂ on the resting potential were studied from the same standpoint and under a variety of experimental conditions. The results and available evidence were found to support the hypothesis.

METHODS

In one group of experiments the procedure described previously (2) was followed. Thus, three pairs of sciatic nerves from *R. pipiens* were mounted at one time in a chamber through which humidified oxygen flowed. In studies of the 'injury potential' the distal ends were crushed and placed in a common trough of 0.111 M KCl; the central intact region of each nerve was put into the end of a solution-filled glass

Received for publication January 16, 1948.

¹ Aided by a grant from the Penrose Fund of the American Philosophical Society.

² A preliminary account of these results was given at a seminar of the Marine Biological Laboratory, Woods Hole, Mass., on July 9, 1946 (37).

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U-tube leading out of the chamber, and the proximal ligated ends were inserted into individual blind tubes containing Ringer. In all gas exchange experiments the central areas of the nerves, after a two-hour soaking period in control and experimental solutions, were raised on filter strips inserted into the U-tubes and their potentials followed for several hours, readings being taken every 15 minutes during at least the last hour to establish a baseline. The potential of the intact region relative to that in KCl is referred to as the 'injury potential' in future discussion. This is considered proportional to the 'resting potential', i. e., the average difference of potential between the axoplasm and medium of the fibers.

In most experiments, including all employing CO_2 , the potential difference between two intact portions of the nerve was recorded. This will be referred to as the 'demarcation potential': The same chamber was used, but the distal ends of the nerves were placed on an insulating ledge and Ringer-soaked filter strips, coming from the trough now filled with Ringer, made contact with the nerves three fourths of a centimeter or more from these ends. Subsequent treatment, including mounting and localized soaking in control and experimental solutions, was the same as described above. The potential changes of the soaked regions relative to the unsoaked areas are given in future discussion.

A final modification for measurement of demarcation potentials consisted of dividing the chamber into two separate compartments. This permitted one half of each nerve—that exposed to control or experimental solution—to be subjected to CO_2 or N_2 while the other half served as a reference in oxygen. Vaseline was used as a seal where the nerves passed through the partition separating the compartments. The effectiveness of the seals was tested by developing sufficient positive air pressure in one compartment to displace the solution or solutions between it and the outside air and then checking for the absence of such displacement in the other compartment. 'Double chamber' will designate this unit, 'single chamber' the absence of such partitioning.

All solutions were made isotonic with 0.111 M NaCl. Unless indicated otherwise, calcium was absent from the experimental media. This was done to minimize the 'stabilizing' action associated with this ion (8). Prolonged soaking in calcium-free Ringer (0.108 M NaCl, 1.7 mM KCl, all-sodium Sørensen phosphate buffer at pH 7.4 and equivalent to 1 mM NaCl) was found to have no detectable deleterious effect on the injury potential; metabolic changes of potential are enhanced (table 8). Experimental substances usually were added to the Ringer by replacement of an osmotically equivalent amount of NaCl. Phosphate buffer concentrations are expressed as the millimols per liter of NaCl to which they are equivalent as found by use of the frog sartorius as an osmometer. Thus, 1.08 gram $\text{Na}_2\text{HPO}_4 + 0.264$ g. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in 100 cc. distilled water is referred to as 0.11 M. Sulfonamide solutions were prepared by the addition of the crystals to Ringer. Since the highest concentration employed was only 100 mgm. per cent (i. e., ca. 6 mM), a hypertonicity of less than 3 per cent was involved. A hypertonicity of 50 per cent was found to have only a slight depressant effect on the demarcation potential (fig. 5A), no effect on CO_2 action, and only a small depressant effect on the anoxic and post-anoxic changes of potential. All concentrations will be given relative to one liter of solution.

Electrical measurements were made at first with a L. and N. Type K potentiometer⁴ and Type R (2500e) galvanometer. Subsequently a Rubicon Type B potentiometer and Type 3415 galva-

⁴ Obtained through the kindness of Dr. R. Keith Cannan and the Department of Biochemistry at the New York University College of Medicine.

nometer (7×10^{-10} A/mm) were employed. With either set of instruments all six potentials were read to within 0.05 millivolt in two to three minutes. Asymmetry potentials in the circuit were checked by replacing the nerves with Ringer-soaked thread.

All gases were taken directly from the tanks. They were humidified by being broken into fine bubbles and passed through a two- to three-foot column of 0.11 M NaCl. Different humidifying columns were employed for each gas to minimize the delay in establishing equilibrium.

RESULTS

Sulfanilamide.^{5, 6} Mann and Keilin (9) describe this as a strong and highly specific inhibitor of carbonic anhydrase. In table 1 its effects are checked against controls with respect to *a*) the demarcation potential, *b*) the anoxic drop in potential,

TABLE 1. CHANGES IN POTENTIAL (MILLIVOLTS) PRODUCED IN PAIRED FROG NERVES BY ANOXIA AND RETURN TO O₂, AND BY 5% CO₂ IN O₂ AND SUBSEQUENT RETURN TO PURE O₂, AFTER 2 HOURS OF LOCALIZED SOAKING WITH CA-FREE RINGER SOLUTION CONTAINING (S) OR LACKING (R) 100 MG. % SULFANILAMIDE

INITIAL DIFF.	INJURY POTENTIAL—SINGLE CHAMBER						DEMARICATION POTENTIAL—SINGLE CHAMBER					
	Fall in N ₂ (1½ hr.)			Recovery in O ₂ (10 min.)			Rise in 5% CO ₂			Fall in O ₂		
S-R	S	R	S/R	S	R	S/R	S	R	S/R	S	R	S/R
2.2	3.2	8.8	0.36	7.3	22.1	0.33	1.5	3.6	0.42	0.8	2.7	0.30
1.5	1.0	4.1	0.24	6.5	18.1	0.36	0.5	1.7	0.29	0.3	0.8	0.38
1.9	8.0	6.2	1.29	4.5	24.1	0.19	1.8	2.8	0.64	1.5	2.2	0.68
1.9	-1.7	7.2		7.2	22.6	0.32	0.4	1.7	0.23	0.0	1.4	0.00
1.8	1.4	2.1	0.67	3.1	14.2	0.22	1.0	3.1	0.32	0.5	1.9	0.24
	-4.1	-2.0		6.8	7.4	0.92						
	4.4	7.0	0.63	4.0	15.0	0.27						
	4.5	10.3	0.44	6.8	18.1	0.38						
M: 1.9	2.1	5.5	0.38	5.8	17.7	0.33	1.0	2.6	0.38	0.62	1.8	0.34
Demarcation Potential—Double Chamber												
M: 1.3(12)	4.49	6.81	0.66(6)	11.35	15.79	0.72(6)	3.2	5.4	0.59(8)	1.63	4.06	0.40(8)

Most N₂ and CO₂ experiments were made on different nerves. Negative values indicate a change opposite in direction to that given by the column title. Parentheses give the number of experiments.

c) the magnitude of the maximum rise in potential upon return to oxygen and *d*) the maximum potential increase in 5 per cent CO₂-O₂ and maximum decrease upon return to O₂. Most experiments were performed with a concentration of 100 mgm. per cent since lower concentrations are less effective and the results correspondingly less consistent.

During anoxia the injury potential of *R. pipiens* sciatic falls for hours (2). Sulfanilamide slows the rate of decline. This is shown by the consistently smaller de-

⁵ Kindly assayed by Dr. Harold A. Frediani, Director of Laboratories of Eimer & Amend, as 97.3% pure. Half the deficit is water, the other half probably degradation products apparently present in other available samples as well.

⁶ Reported at the thirtieth annual meeting (March 15, 1946) of the Federation of American Societies for Experimental Biology (36).

crease (or, in some instances, greater rise) in potential after $1\frac{3}{4}$ hours of anoxia. The apparent rise during anoxia obtained with some of the nerves is probably the result of a potential drift exceeding the normal anoxic decline. Thus, four hours after mounting the injury potential usually is slowly and asymptotically rising to a plateau (4). The change of potential in nitrogen, if measured as the difference in injury potential just before and after anoxia, would be smaller than if allowance were made for the rise. Correction for this drift is possible by log.-log. plotting of the potentials initially obtained in oxygen. A straight line usually is obtained, providing an apparently reliable baseline for extrapolation into the anoxic period. This assumes, however, that drift is the same during anoxia. For comparative purposes the extrapolation is unnecessary when control and experimental nerves are taken from the same animal. As may be seen in the nitrogen experiments of table 1, data variability among nerves from different animals is considerable; nevertheless, the ratio S/R (millivolts change in sulfanilamide relative to that of the controls) or comparison of the direction and magnitudes of the potential changes shows that in 13 of the 14 *paired* nerves sulfanilamide retards the anoxic decline of potential.

The rise in injury potential during post-anoxic recovery in oxygen usually is complete in less than 10 minutes, is larger than the anoxic fall, and is followed by a slow decline. Sulfanilamide reduces the amplitude of the rise (table 1) without perceptibly altering the rates or sequence of the potential changes. The rapidity of the rise makes drift a negligible factor. Nevertheless there still is considerable variability among unpaired nerves. Such variability therefore may be related to the large differences in respiratory rate among nerves from different frogs (7). The good agreement in respiratory rates between paired nerves (7) would explain the almost 100 per cent consistency of data obtained with pairing in these and subsequent experiments.

Measurements with the demarcation potential and the double chamber confirm the results obtained with the injury technique (table 1).

The question may be raised as to whether the reduced recovery in sulfanilamide merely reflects the slowed decline in potential during anoxia (i. e., the small initial fall requires a smaller rise for a return to normal) or whether the drug has a specific effect on the recovery process itself. The latter is suggested by one experiment in table 1 in which the potential during anoxia actually fell further in sulfanilamide and yet the recovery upon return to oxygen showed the greatest relative impairment. This conclusion also is supported by experiments with nerves subjected to iodoacetate and pyruvate. Such preparations maintain their potentials for long periods, respond more rapidly than Ringer controls to anoxia and, if exposure is not too prolonged, recover readily in oxygen (2). The nerves of table 2 were exposed for $3\frac{1}{2}$ hours to both iodoacetate and pyruvate, with sulfanilamide either present or absent. Under these conditions sulfanilamide delays the anoxic decline only initially, the potential change after 100 minutes being practically the same for controls and experimental nerves. Nevertheless the potential rise upon return to oxygen is less in sulfanilamide. As in the preceding experiments, the time course of recovery of these preparations is not altered perceptibly by the sulfonamide.

Previous inhibition of oxygen consumption, associated with a lowering of in-

jury potential, also may reduce the absolute changes of potential during anoxia and recovery. This is obtainable by prolonged exposure of nerves to iodoacetate and fluoride (2). However, in contrast to the results with these inhibitors, long exposure (39 hours) to sulfanilamide gives injury potentials decidedly superior to the controls. Furthermore, the demarcation potential of nerves subjected for three to four hours to sulfanilamide averages about 1.5 millivolts higher than in the controls. This is shown by the consistently positive values obtained by subtracting the demarcation potentials obtained with Ringer from those with sulfanilamide at the end of baseline runs (table 1).

As a test for the involvement of carbonic anhydrase the effect of sulfanilamide on the action of CO_2 also was examined. Five per cent CO_2 in oxygen was the only mixture used. Because of the small potential changes produced, only the demarcation potential was employed.

In preliminary experiments the entire nerves were subjected to CO_2 . The region which had been soaked in the U-tube invariably showed a rapid rise in potential relative to the area closer to the distal end, although both were on filter strips and both were in the CO_2 (table 1 and fig. 1A). This rise as well as fall upon return to oxygen is less in sulfanilamide.

When the action of CO_2 is limited to the experimental region of the nerve by use of the double chamber, the magnitude of the potential changes is doubled (table 1). This is to be expected from the elimination of the potential rise at the reference electrode. Figure 1B is typical of the records obtained: The application of CO_2 produces a rapid initial rise followed by a secondary slower fall. Two such phases of CO_2 action were described previously by Necheles and Gerard (10). As in the single chamber, the maximum rise in potential and the fall upon return to pure oxygen are reduced by sulfanilamide (table 1, fig. 1B). Like post-anoxic recovery, however, the time course of potential change is unaltered.

The smaller rise of potential in the presence of sulfanilamide is attributable to two factors. One possibility is that the elevation of potential in 5 per cent CO_2 is limited to a certain maximum and sulfanilamide, by initially raising the potential closer to this value, restricts the amplitude of the response. In keeping with this, the initial difference in demarcation potentials (S-R column of table 1) is about the same as the difference in the potential changes in CO_2 of Ringer and sulfanilamide-soaked nerves. Another possibility is that the initial rise in CO_2 is slowed beyond detection by the slow potentiometric procedure employed but sufficiently for the secondary decline to cut off the rise at a lower level of potential. Such an effect by the secondary decline is apparent in figure 3.

*Thiophene-2-sulfonamide.*⁷ Davenport (11) has described thiophene-2-sulfonamide as a stronger inhibitor of carbonic anhydrase than sulfanilamide. It is therefore of interest that a lower concentration of this sulfonamide consistently produces the same effects with regard to demarcation potential changes in CO_2 and during anoxia and post-anoxic recovery (table 3). The last two sets of data also demonstrate a remarkable degree of correlation between the magnitudes of the potential changes of paired

⁷ Dr. R. O. Roblin, Director of the Chemotherapy Division of the American Cyanamide Corporation, very generously supplied this material.

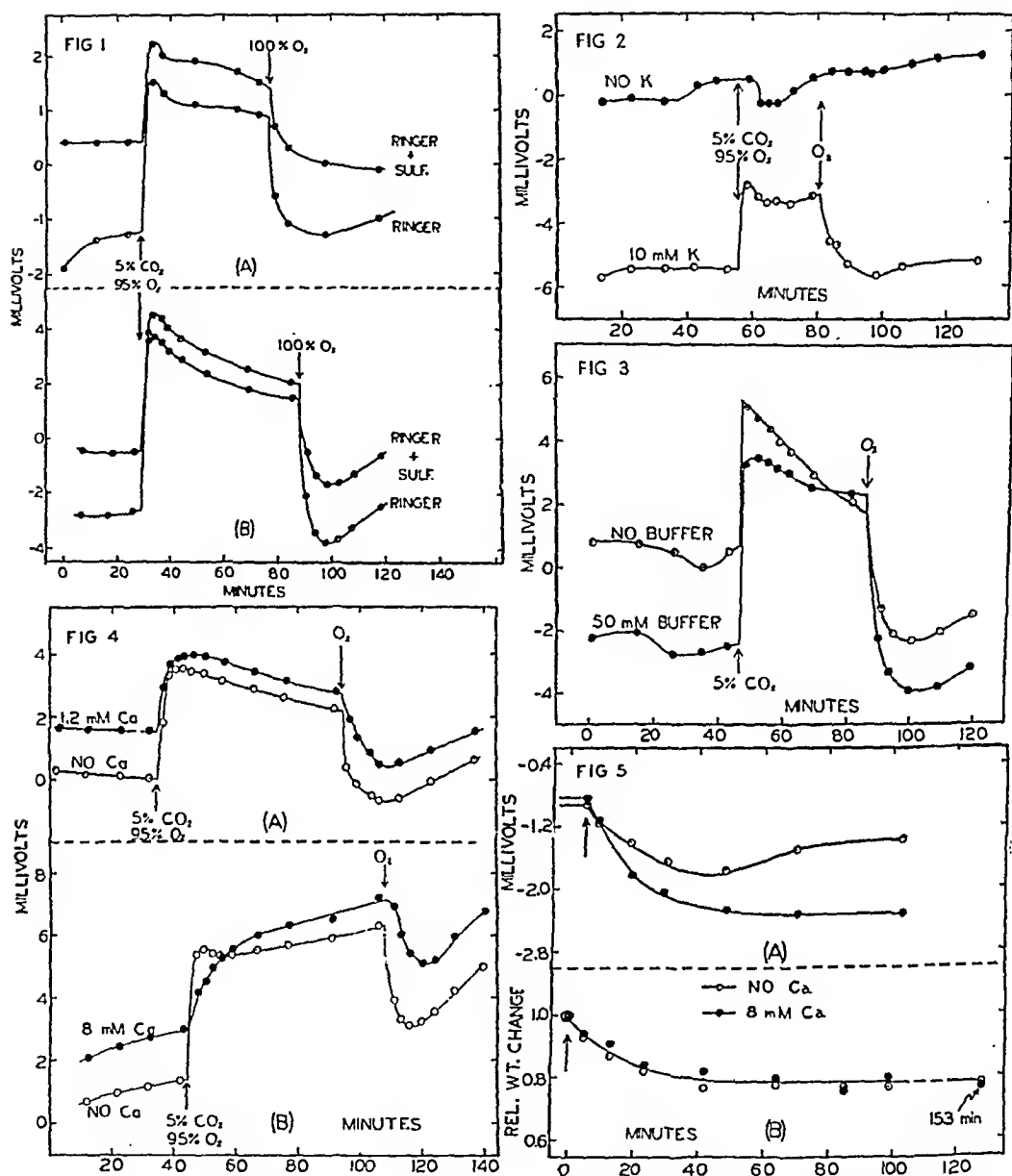


Fig. 1. TYPICAL POTENTIAL CHANGES when 5% CO₂ is applied to sciatic nerves previously soaked for 2 hours in Ca-free Ringer. The effect of 100 mgm. % sulfanilamide is also demonstrated. A, single chamber; B, double chamber.

Fig. 2. EFFECT OF THE ABSENCE OF K in the extracellular spaces on the action of CO₂ on the demarcation potentials. Strong phosphate buffering (25 mM).

Fig. 3. COMPARISON OF STRONG AND NO EXTRACELLULAR PHOSPHATE BUFFERING on the action of CO₂ on the demarcation potential.

Fig. 4. EFFECT OF CA on the action of CO₂ on the demarcation potential.

Fig. 5. A. EFFECT OF HYPERTONIC RINGER, 1.5 × normal tonicity by the addition of sucrose, on the potential of the local region to which it is applied. Each point is the average of 3 experiments. B. Effect of hypertonic Ringer, 2 × normal tonicity by the addition of sucrose, on the weight of sciatic nerves relative to their initial weight. Arrows indicate the change to hypertonic solution.

nerves over the wide range of values obtained. This follows, too, from the remarkable consistency of the results. The fourth and fifth pairs of nerves from the top of

the anoxia series in table 3 provide further evidence for the action of the sulfonamide specifically on the recovery process apart from its effect on the anoxic decline (c.f. table 2).

Davenport's results might lead one to expect greater effectiveness from thiophene-2-sulfonamide than actually observed. However, if carbonic anhydrase is present in very small quantities in nerve, it would be practically saturated by both inhibitors. Interference by the membrane with ionic transport may be another limiting factor (32). A third possibility is that these sulfonamides are acting in a less specific fashion like cocaine and procaine (12) to reduce membrane permeability.

TABLE 2. ANOXIC DECLINE AND RECOVERY IN O_2 (MV) OF THE INJURY POTENTIAL OF NERVES EXPOSED TO CA-FREE RINGER CONTAINING 0.002 M IODOACETATE AND 0.02 M PYRUVATE (IP), WITH 100 MGM. % SULFANILAMIDE (S) PRESENT OR ABSENT

FALL IN N_2									RECOVERY IN O_2		
At 30 min.			At 60 min.			At 100 min.			At 20 min.		
IPS	IP	IPS/IP	IPS	IP	IPS/IP	IPS	IP	IPS/IP	IPS	IP	IPS/IP
6.8	10.3	0.66	12.2	15.3	0.80	14.9	15.7	0.95	12.9	16.6	0.78
4.5	8.1	0.56	9.1	10.3	0.88	10.1	10.4	0.97	10.7	13.9	0.77
5.1	5.2	0.98	7.9	7.8	1.01	8.9	8.4	1.06	8.4	14.8	0.57
M:		0.73			0.90			0.99			0.71

TABLE 3. EFFECT OF 50 MGM. % THIOPHENE-2-SULFONAMIDE (I) IN CA-FREE RINGER, COMPARED WITH THAT OF CA-FREE RINGER (R), ON THE RESPONSE (MV), OF THE DEMARCATION POTENTIAL UNDER DIFFERENT GASEOUS CONDITIONS

RISE IN CO_2			FALL ON RETURN TO O_2			FALL AFTER 60 MIN. IN N_2			RECOVERY IN O_2		
I	R	I/R	I	R	I/R	I	R	I/R	I	R	I/R
3.1	3.8	0.82	2.25	2.95	0.76	14.4	20.8	0.69	15.5	25.0	0.62
3.1	3.65	0.85	1.9	2.3	0.83	9.6	12.0	0.80	15.0	22.3	0.67
1.7	4.2	0.41	1.3	2.95	0.44	5.4	9.9	0.55	5.3	8.9	0.60
2.5	3.6	0.70	2.15	3.2	0.67	3.0	2.3	1.30	7.7	10.2	0.76
1.5	1.9	0.79	0.95	1.55	0.67	8.6	8.9	0.97	13.8	17.9	0.77
						7.0	10.7	0.65	14.2	18.5	0.77
M: 2.4	3.4	0.71	1.7	2.6	0.65	8.0	10.8	0.74	11.9	17.1	0.70

Sulfanilic acid and sulfapyridine are lacking in carbonic anhydrase activity (9). In three experiments tried with each substance (110 mgm. per cent and saturated solutions respectively) no effect in either CO_2 or anoxia and post-anoxia was observed.

Potassium. The foregoing results are in keeping with the possibility that carbonic anhydrase and hydrogen ions are involved. This and the involvement of potassium may be tested further by appropriate modification of the ionic medium.

Thus, if the uptake of potassium is the primary cause of the potential rise in CO_2 , this rise should be prevented in the absence of potassium. Such an effect is demonstrated in figure 2. These nerves had been soaked in a potassium- and calcium-free Ringer for 16 hours at 4°C. and then mounted at room temperature with

the experimental regions soaking for two more hours in either the potassium-free solution or, as a control, a solution containing 10 mM potassium. They underwent the usual changes in potential upon subsequent exposure to nitrogen and return to oxygen.

The length of the preliminary soaking period in such potassium experiments must be emphasized as of great importance. Nerves taken from the same batch of spring frogs as used for figure 2 showed no such effect after only several hours of soaking in potassium-free solution; in fact, the response to CO_2 was slightly and significantly higher. This is explained by the observation by Fenn *et al.* (13) that under these conditions potassium leaks from the fibers into the extracellular spaces for many hours, an effect which would prevent adequate depletion of extracellular potassium over short soaking periods. The finding by Fenn *et al.* (13) that autumn nerves leak potassium less readily than nerves taken from spring animals also would explain

TABLE 4. COMPARISON OF MAXIMUM DEMARCATION POTENTIAL CHANGES (MV) INDUCED BY 5% CO_2 PRIOR TO AND 45 MIN. FOLLOWING 2 HOURS OF ANOXIA

CO_2 PRIOR TO ANOXIA		CO_2 FOLLOWING ANOXIA	
Rise in CO_2	Fall on return to O_2	Rise in CO_2	Fall on return to O_2
3.2 ¹	2.3 ¹	0.25 ¹	0.3 ¹
4.3	4.0	0.65	1.1
4.2 ¹	3.2 ¹	0.15 ¹	0.8 ¹
7.2	5.9	1.25	2.4
2.8 ¹	3.5 ¹	0.50 ¹	1.5 ¹
4.2	4.3	1.60	2.9
<i>M</i> : 4.3	3.87	0.77	1.5

Soaking medium: Ca-free Ringer's solution.

¹Sulfanilamide, 100 mgm.%, in Ca-free Ringer's solution.

why only two hours of soaking sufficed to halve the CO_2 response in every one of three experiments performed earlier with winter nerves.

The change in demarcation potential during post-anoxic recovery usually is greater than the decline in nitrogen (14). If these potential changes represent shifts in potassium, the greater rise during recovery must deplete the extracellular spaces of this ion. Consequently the effectiveness of CO_2 during the recovery phase might be less than before anoxia. Table 4 gives the magnitudes of the demarcation potential changes produced by CO_2 during these two periods. The effectiveness of CO_2 is markedly reduced during recovery, the differences of the means under the two conditions being better than three times the standard deviations of the differences.

Buffering. The secondary decline of potential in CO_2 may be due to the rise of the hydrogen ion concentration in the weakly buffered extracellular spaces, a rise which would be slower than within the fibers for lack of carbonic anhydrase. The effect of different extremes of phosphate buffering of the medium therefore was examined. As may be seen in figure 3, the complete absence of buffer increases the

secondary decline markedly while very strong buffering which limits the pH change to a few tenths of a unit is associated with only a slight secondary decline, the general level of potential being well maintained. The latter suggests that a permanent uptake of potassium occurs in CO₂ with strong buffering, a conclusion supported by the analytical measurements of Fenn and Cobb (15).

It is obvious in figure 3 that the rate of secondary decline is a controlling factor

TABLE 5. EFFECT OF STRONG EXTERNAL PHOSPHATE BUFFERING (P), 25 OR 50% OF ISOTONIC STRENGTH IN CA-FREE RINGER, COMPARED WITH THAT OF LOW (1 mM PO₄) OR NO BUFFERING IN CA-FREE RINGER (R), ON THE DEMARCATION POTENTIAL (MV) OF FROG NERVE UNDER DIFFERENT GASEOUS CONDITIONS

CHAMBER	INITIAL POTENTIAL DIFF.	RISE IN CO ₂		FALL ON RETURN TO O ₂		CHAMBER	FALL AFTER 100 MIN. IN N ₂		MAX. RISE ON RETURN TO O ₂	
		P	R	P	R		P	R	P	R
A	-1.4	2.6	1.6	2.9	1.3	A	4.1	3.90	20.2	17.1
A	-2.5	3.2	1.9	3.1	2.3	B	7.6	3.05	21.0	11.9
A	-3.0	5.9	4.5	6.2	4.3	B	8.5	2.20	23.0	11.2
B	-2.4	4.8	1.4	2.0	0.1	B	7.4	7.55	18.7	21.0
B	-2.0	5.4	2.3	4.0	1.8					
B	-2.9	6.1	2.4	6.1	0.9					
M:	-2.4	4.7	2.4	4.1	1.8		6.9	4.2	20.7	15.3
P/R		1.95		2.28			1.65		1.35	

A—Double chamber; B—Single chamber.

TABLE 6. EFFECT OF 0.01M CITRATE (C) IN CA-FREE RINGER ON THE CHANGE IN DEMARCATION POTENTIAL (MV) COMPARED WITH THAT OF CALCIUM-FREE RINGER (R) UNDER DIFFERENT GASEOUS CONDITIONS

INITIAL POTENTIAL DIFF.	RISE IN CO ₂		FALL ON RETURN TO O ₂		FALL AFTER 75 MIN. IN N ₂		RECOVERY IN O ₂	
	C	R	C	R	C	R	C	R
-2.3	2.85	4.30	2.30	3.95	11.7	6.7	24.1	16.8
-0.95	0.70	2.40	0.23	2.00	7.4	5.7	19.7	14.3
-2.45	3.35	5.55	3.67	4.90	5.7	-2.2	17.1	12.3
M: -1.90	2.30	4.1	2.1	3.6	8.3	3.4	20.3	14.5
C/R	0.56		0.58		2.44		1.4	

in the amplitude of the potential rise in CO₂. From this standpoint the consistently greater effectiveness of CO₂ with strong buffering (table 5) provides supplementary evidence of a reduced secondary decline.

A serious limitation in the use of phosphate as a buffer is its calcium-combining property. Chang *et al.* (16) have demonstrated that calcium precipitants increase oxygen consumption, citrate in 10 per cent of isotonic strength being more potent than an isotonic solution of phosphate. The action of 0.01 M sodium citrate therefore was examined (table 6). It is apparent citrate and phosphate are alike insofar

as they lower the potential at the locus of application and, as might be expected from increased respiration, enhance the excursions of potential during anoxia and recovery. These effects consequently are attributable to their ability to combine with calcium. Citrate, however, reduces rather than increases the effectiveness of CO_2 , presumably for lack of the buffering action of phosphate.

The effect of potassium in the medium on the small secondary fall in potential invariably present with strong buffering also was examined. Attention to the baseline prior to the application of CO_2 was of special importance in these experiments. In table 7 the absolute secondary changes in potential as well as the changes relative to the initial rise are compared in spring nerves which were previously soaked for two hours in either a potassium-free or 5 mM potassium solution. Although the potential changes are small, the absence of potassium consistently reduces the secondary decline. This may explain the small increase in the initial potential rise in CO_2 .

TABLE 7. EFFECT OF 5 mM OF K IN THE BATHING MEDIUM ON MAGNITUDE OF THE SECONDARY DECLINE IN DEMARCATION POTENTIAL (MV) 45 MINUTES FOLLOWING THE INITIAL MAXIMAL RISE IN RESPONSE TO 5% CO_2

DECLINE IN K	DECLINE OF CONTROL	DECLINE IN K INITIAL RISE	CONTROL DECLINE INITIAL RISE
1.55	0.30	0.82	0.14
1.65	0.65	0.55	0.19
0.80	0.0	0.62	0.0
1.25	0.35	0.21	0.05
2.25	0.75	0.46	0.16
M: 1.50	0.41	0.53	0.11
Na/K 0.27			0.21

Controls (Na) were soaked for the same period (2 hours) in a K-free solution.

previously noted for spring nerves exposed for short periods in potassium-free solution.

The results in table 7 may be accounted for by considering the extracellular space to consist of two parts, one in good contact with the fibers and the other separated from the fibers by the dense sheathing material characteristic of the nerve (7). The initial rapid uptake of potassium in CO_2 would be restricted to the former, but some potassium would reenter this space from that more remote, causing the potential to fall again. The existence of a double extracellular space also is suggested by the significantly greater weight changes of nerves placed in a Ringer made hypertonic with sucrose than when NaCl or KCl are the hypertonic agents (unpublished).

Calcium. The property of this ion to decrease permeability has been demonstrated repeatedly (17). Thus, Fenn (18) has shown that calcium delays potassium loss in frog nerve. Consequently it would be expected to delay ionic exchange.

Figure 4 is typical of the action of calcium observed in all CO_2 experiments. It slows the rise of potential even at very low, physiological concentrations, as may be seen from the greater length of time required for the potential to reach a maxi-

num. The effectiveness of calcium is proportional to the concentration. The demarcation potential was usually higher in calcium.

Table 8 summarizes the data on the effect of calcium on the potential changes in CO_2 and during post-anoxic recovery. Both are reduced. The available data suggest a greater effect on the latter as calcium is increased, in keeping with the effect of calcium on respiration (16). However, no effect on the time course of post-anoxic recovery could be noted.

Hypertonicity. Another test for the involvement of potassium has been suggested by Wilbrandt (19), viz., increase of the intracellular potassium concentration. His results, obtained by making Ringer hypertonic with glucose, did show an increase in potential but, according to an example given, this rise was not complete after six hours in the hypertonic medium.

An earlier attempt to duplicate Wilbrandt's results with sucrose instead of glucose caused a slight fall rather than a rise in potential (5). This action of sucrose has been observed again and the time course of the potential decline followed (fig. 5A).

TABLE 8. ACTION OF VARIOUS CA CONCENTRATIONS IN RINGER ON THE RESPONSE OF THE DEMARCATION POTENTIAL TO DIFFERENT GASES COMPARED WITH THAT OF CA-FREE CONTROL SOLUTIONS

CA IN MM	$\text{O}_2 \rightarrow \text{CO}_2$	$\text{CO}_2 \rightarrow \text{O}_2$	$\text{N}_2 \rightarrow \text{O}_2$	CA IN MM	$\text{O}_2 \rightarrow \text{CO}_2$	$\text{CO}_2 \rightarrow \text{O}_2$	$\text{N}_2 \rightarrow \text{O}_2$
1.2	0.70	0.79	0.50	8.0	0.60	0.67	0.36
3.2	0.57	0.79	0.60	8.0	0.46	0.82	0.52
3.2	0.77	0.85	0.73	24.0	0.69	1.11	0.22
Mean	0.68	0.81	0.61		0.58	0.87	0.37

Values given are the ratio of potential change with Ca to that in the absence of Ca.

A similar decline has been observed with glucose as well, whether or not calcium or potassium is present up to 10 per cent of isotonicity. Since this decline is complete in about an hour, in contrast to Wilbrandt's observations, it was desirable to check on the time course of the osmotic response.

The osmotic behavior was determined by following the weight of individual sciatic nerves. After several hours equilibration in Ringer a control series of weighings was made and the nerves subsequently subjected to the hypertonic solution. For each weight determination the nerves were carefully blotted and then rapidly weighed with a Chain-o-matic precision balance. Threads fastened to the ends of the nerves were used for handling, their weights being determined and allowed for at the end of the runs. The reproducibility of the data so obtained was very good, the standard deviation of the mean for six preparations being 0.6 to 0.8 per cent as compared with the maximum weight change of more than 20 per cent. Action potentials were unaffected in hypertonic solution and upon return to Ringer, indicating the absence of damage to the fibers.

Figure 5B demonstrates the time course of weight change in 2x hypertonic solution (sucrose equivalent to 0.111 M NaCl added to Ringer). Like the drop in potential, equilibrium is attained in about an hour. It must be concluded that Wil-

brandt's results, although more desirable for the thesis being tested, were not due to an increase in intracellular potassium concentration. An explanation of his observations is seen in his procedure for mounting the nerves: the interelectrode regions were immersed in mercury and therefore undoubtedly anoxic. If glucose can provide energy for maintaining the potentials under anoxic conditions, the protracted rise of potential he observed would follow from the diffusion of glucose into these areas. Evidence for such a metabolic effect by glucose has been obtained.

Glucose. Table 9 summarizes the effect of glucose and sucrose, added to Ringer in osmotically equivalent amounts, on the anoxic decline and post-anoxic recovery of potential at the locus of application. The decline in nitrogen is negligible in glucose, even when allowance is made for drift, and the recovery in oxygen is correspondingly small. The potential changes observed with the sucrose controls, however, are much larger and of the magnitude obtained with other controls.

TABLE 9. EFFECT OF 1.87% GLUCOSE, IN CA-FREE RINGER, COMPARED WITH THAT OF AN EQUIVALENT OF SUCROSE (3.55%) ON THE ANOXIC DECLINE AND THE RECOVERY IN O₂ (MV) OF THE DEMARCATION POTENTIAL

FALL IN N ₂ (AFTER 1 HOUR)		RECOVERY IN O ₂ (MAX., AT 7 MIN.)	
Glucose (G)	Sucrose (S)	Glucose	Sucrose
0.65	16.35	1.50	23.10
-0.55	7.80	1.85	13.60
-1.70	6.00	6.45	15.05
-2.00	5.80	2.90	10.10
M: -0.9	9.00	3.18	15.5
G/S	-0.1	0.205	

The effectiveness of glucose in reducing the potential changes associated with anoxia provides a specific basis for explaining the variability of the data among nerves from different animals. Thus, the differences may have been due to different amounts of glucose retained in the extracellular spaces. This retention would depend on (1) the nutritional state of the animal at the time of dissection, (2) nerve thickness, (3) permeability of the sheaths and (4) duration of the soaking period. The better agreement between nerves from the same animal would follow from the better control of these factors.

DISCUSSION

The various indirect experiments with CO₂ which have been described, particularly in the light of direct chemical evidence for potassium movement under one of the conditions, strongly favor the involvement of hydrogen-potassium exchange. The increased production of CO₂ during post-anoxic recovery (21) is presumptive evidence that the potential change during this period is mediated at least in part by the same fundamental mechanism. In keeping with this it has been shown that the potential changes during both recovery and CO₂ exposure are affected similarly by inhibitors of carbonic anhydrase. The reduced responsiveness of nerves to CO₂ during the post-anoxic period is also in accord with this view.

A correlation between the potential changes in CO_2 and during post-anoxic recovery was also observed with phosphate buffer and calcium. However, the effect of these agents on recovery, as in the case of citrate, is related more obviously to their action on respiratory rate (16) than to the ionic exchange rate. This is probably because during recovery the return of CO_2 production rather than ionic exchange is the limiting factor. The following considerations suggest, nevertheless, they are not unrelated.

The removal of calcium from the medium, accentuated by calcium precipitants, may be imagined to increase the tendency of potassium to escape from the fibers. The existent respiratory rate and associated rate of hydrogen ion formation is now inadequate to retain the potassium, resulting in some loss with an accompanying depolarization. As this loss progresses, a compensatory mechanism increases the rate of respiration and thereby increases the rate of hydrogen ion formation to the point where potassium leakage ceases. A new steady state will have been achieved. An increase in calcium would reverse this sequence. If this approach is correct, calcium has the important function of conserving the energy resources of the cell.

In accord with this view are *a*) the established inverse relationship between respiratory rate and calcium concentration (16), *b*) the greater loss of potassium in the absence of calcium (18), and *c*) the consistent lowering of potential by calcium precipitants (tables 5 and 6). The greater excursions of potential observed during anoxia and post-anoxic recovery with low calcium (tables 5, 6, and 8) would follow from the greater dependence of the potassium level (and hence the potential) on respiration. The 'stabilizing' action of calcium against a wide variety of potential lowering agents (21), including anoxia (8), may represent merely a decreased rate of potassium leakage. The same basic action of calcium on ionic movement can explain the ability of this ion to prevent the lowering of potential by other externally applied ions such as potassium. Thus, Höber (22) points out from the standpoint of membrane diffusion potentials that reduced mobility in the cell membrane will decrease the effect of the ions on the potential.

If the anoxic decline of potential is due to the failure of hydrogen ion production, a substrate like glucose, which has been demonstrated to maintain a high rate of lactic acid formation in nerve (23), should prevent the fall, as already demonstrated. Earlier observations (2) support this conclusion and point specifically to the hydrogen as the ion involved. Thus, when lactic acid production is blocked with iodoacetate or fluoride the anoxic decline of potential is accelerated; high concentrations of sodium lactate in the medium do not modify the anoxic decline whether the inhibitors are present or not. The ability of lactate to counteract iodoacetate under aerobic conditions demonstrates that the negative effect under anoxic conditions is not due to impermeability; it also demonstrates a utilization at the lower end of the glycolytic cycle, presumably decarboxylation to form CO_2 , under aerobic conditions. The earlier suggestion (2) that phosphorylations are directly associated with potential production is not completely ruled out, but in the light of the additional evidence this is considered to be secondary at best to the hydrogen ion mechanism.

Older observations suggest that potassium does not leave nerve or muscle fibers during anoxia. However, in those instances in which the techniques are described the results are found to be vitiated by the procedure employed. Thus, Dean (24)

returned frog muscles to air for the purpose of weighing them before beginning the analyses. Cowan (25), on finding that excitability returns in crab nerves restored to oxygen following a bout of anoxia concluded that potassium had not accumulated around the fibers previously. Both investigators make the questionable assumption that an anoxic loss of potassium is permanent. Fenn (18) has not described his methods, but his finding of a greater potassium concentration in frog nerve after anoxia suggests that recovery occurred in these experiments as well; as already pointed out, the different magnitudes of the potential changes during anoxia and post-anoxic recovery indicate a greater potassium shift in the latter phase.

Aside from the questionable procedures employed, the significance of analytical studies of potassium movement must be considered from the standpoint of the magnitude of the shifts involved. An estimate may be made by applying the formula for a membrane concentration potential, a procedure justified by the demonstrated linearity between the injury potential and the logarithm of the extracellular potassium concentration (5). Since the nerves were mounted in air, any potassium lost from the fibers would accumulate around them. As a first approximation the volumes of the intracellular and extracellular spaces may be assumed equal, hence any change, X , in concentration of the former will be equal but opposite to that in the latter.

The injury potential, E , therefore would be governed by $E = C \log \frac{K \pm X}{k \pm X}$. K and k are the initial intracellular and extracellular potassium concentrations and C is taken as 30 millivolts instead of 58 to allow for short-circuiting; k usually is small, consequently small values of X will have a large effect on the ratio and E . For example, anoxia may lower the potential about 10 millivolts in an hour. Taking E as 40 millivolts and k as 2 mM, K is 45 mM and X , for the anoxic decline, is only 2 mM. Obviously, although the potential changes are large the potassium shifts may require considerable precision for determination. Gross analysis, of course, would indicate no change at all since under these conditions all potassium movement is restricted to the nerve trunk. On the other hand, if the fibers were in perfect contact with a large amount of solution, the calculation shows that a 4 per cent concentration change would have to be detected. Actually, of course, the dense sheaths of the nerve would reduce the loss.

The hypothesis of potassium-hydrogen exchange requires that the fiber membranes be permeable to hydrogen and potassium ions but impermeable (or relatively so) to sodium and anions. The potential and osmotic experiments by Netter (26, 27) indicate such is the case in frog nerve. Yeast, which has similar membrane properties, unquestionably undergoes such exchange (28, 29). It is important to note that the acids providing hydrogen ions are not necessarily the same in different systems, which is undoubtedly related to fundamental biochemical differences. Membrane properties may also be a factor. Thus, the permeability of crab nerve to anions appears to account for the different relationship of metabolism to the resting potential (30, 31).

One surprising feature of the results is the absence of a perceptible effect by carbonic anhydrase inhibitors on the *rate* of rise of potential in CO_2 and during post-anoxic recovery. Ionic exchange and the rate of CO_2 production by the nerve itself

may contribute to this by being the limiting factors in the potential change. Booth (32) has found the former to limit the effectiveness of carbonic anhydrase in the intact red cell. The initial elevation of potential in sulfanilamide may indicate a condition also of importance in this regard. For example, such an increase in potential would result if, under resting conditions, hydrogen and bicarbonate ions rather than CO_2 were the first products of decarboxylation; sulfanilamide, by delaying the formation of CO_2 from the ions, would increase the hydrogen ions available for exchange and simultaneously raise the buffering capacity of the cells. The effect on buffering would account for the reduced response of the potential to applied or metabolically produced CO_2 . The observation that cocarboxylase does give rise to hydrogen and bicarbonate ions first (33) lends credence to this view, but more evidence certainly is to be desired.

Another surprising result is the failure of the potential to rise when the intracellular potassium concentration was increased with hypertonic solutions. This, however, is not necessarily evidence against the potassium gradient hypothesis. Thus, the injury potential of muscle (34) and invertebrate nerve (35, 36) cannot exceed a certain maximum regardless of the steepness of the potassium gradients. This is associated with leakage of potassium from the fibers (34, 13). The steeper gradient produced with the hypertonic solutions also may have resulted in leakage, the rise in extracellular potassium thereby preventing the anticipated potential elevation. In any event, the action of hypertonic solutions will require further study before the relation of these results to the potassium concentration hypothesis can be fully evaluated.

In view of the available evidence, the hypothesis of hydrogen-potassium exchange is offered as a necessary and sufficient explanation of the potential changes under conditions of anoxia and post-anoxic recovery as well as in CO_2 .

SUMMARY

Sulfanilamide and thiophene-2-sulfonamide slow the decline of potential in frog nerve during anoxia and reduce the rise during post-anoxic recovery. Glucose prevents the anoxic decline. Five per cent CO_2 , which produces a rapid initial rise followed by a slower fall in potential, is less effective in the sulfonamides. The CO_2 response is modified by the medium around the fibers. Thus, in the absence of potassium it is prevented, calcium slows the initial rise, and strong phosphate buffering reduces the secondary decline. From these and other observations it is concluded that the potassium gradient is at least an important source of the resting potential in frog nerve, and that metabolism contributes by providing hydrogen ions which, through exchange with extracellular potassium, serve to maintain this gradient.

It is a pleasure to acknowledge my indebtedness to the many investigators at Woods Hole and to Dr. F. Brink who read and commented on the original manuscript, and to Dr. D. E. S. Brown for his stimulating interest during the course of this investigation.

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EFFECT OF REPEATED DENERVATION UPON NEUROMUSCULAR FUNCTION¹

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THIS investigation was prompted by reports (1, 2) that functional recovery of partially denervated skeletal muscle could be enhanced by increasing the degree of reinnervation through branching of remaining viable axons, a process known as 'neurotripsy.' These reports, together with the observation (3-6) that a certain amount of spontaneous functional recovery occurs in partially denervated muscle through the operation of peripheral mechanisms which do not involve reinnervation of paralyzed muscle fibers by regenerating motor fibers, made it seem of interest to study the effect of repeated cycles of denervation and reinnervation upon neuromuscular function.

The experimental procedures which were employed in this study were similar to those described in a previous report (7). All operative procedures were done under ether anesthesia. Total denervation of the gastrocnemius muscle of the rat was accomplished by crushing the tibial nerve at its junction with the peroneal with a heavy linen ligature against a brass rod. The nerve is all but severed by this procedure and the traumatized segment is soon filled with blood. The repeated crushings of the nerve were performed as near the same location as possible. Forty-two days of recovery were allowed to elapse between crushing operations. In one group of animals the nerve and muscle of the unoperated contralateral limb served as a control. In another group the experimental limb was subjected to two or three nerve crushes at 42-day intervals and was matched against controls which had been denervated once. Studies of the weight and strength of the control and experimental gastrocnemii were made at 21, 42 and 84 days after the final denervation. For measurements of muscle strength the tendon of Achilles was cut and attached to a Blix-type torsion rod and the femur was fixed in a rigid clamp. The muscle was stimulated directly by volleys of slightly supermaximal stimuli applied to the muscle through needle electrodes and indirectly by volleys of supermaximal condenser discharges delivered to the tibial nerve through adjustable silver electrodes. The frequency and strength of the electrical shocks were such as to evoke maximal isometric tetanus tension responses in the muscle. The extent of muscle shortening was measured from optical records and the strength was considered to be the maximal tension which developed in response to either direct stimulation of the muscle or of its motor nerve. The tension responses of nondenervated muscle to direct and indirect stimulation were approximately equal under these conditions. At the conclusion of the strength measurements the gastrocnemii were dissected out and weighed.

A total of 120 female albino rats of the Sprague-Dawley strain, closely matched as to age and body weight, was used in this study. Care was taken to match the animals as to age at the time of the final denervation operation when the effects of repetitive denervations were compared with the effects of single denervations.

RESULTS AND DISCUSSION

The data and calculations presented in figure 1 and table 1 take into consideration that about 15 per cent of the weight of the normal gastrocnemius muscle of the rat consists of nonmuscle cell phase and 85 per cent represents muscle cell phase (8). The nonmuscle cell fraction is made up of connective and nerve tissue, blood vessels,

Received for publication March 8, 1948.

¹ Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

etc. and is not directly concerned with tension development. The absolute amount of this phase is essentially unaltered during atrophy and regeneration. The changes in weight which a muscle undergoes during atrophy and regeneration are chiefly related to changes in the amounts of muscle cell phase. It has been shown (9) that muscle weight loss after a single denervation follows the equation of a reaction of the first order, i.e., weight loss at any given time is a constant fraction of the losable weight existing at that time. It is apparent that different ratios exist for muscle cell and nonmuscle cell phases at 42 days after denervation, a time at which regeneration is incomplete, than those found in normal control muscle. These considerations made it desirable to express the changes in muscle weight and strength on the basis of muscle cell phase rather than upon total muscle weight. The data on the effects of repeated denervations were analyzed on the assumption that the degree of

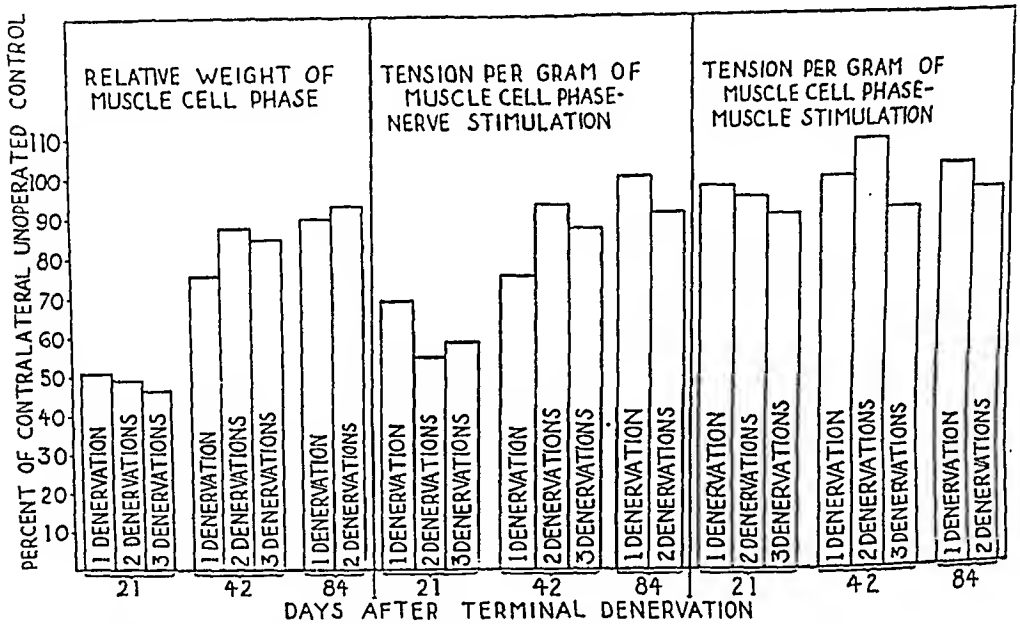


FIG. 1. EFFECTS OF REPEATED DENERVATION ON THE WEIGHT AND STRENGTH OF THE GASTROCNEMIUS MUSCLE OF THE RAT.

atrophy and subsequent regeneration would be influenced by the amount of muscle cell phase present at the time of any denervation.

Measurements made at 21 days after denervation (fig. 1, table 1) show that after one, two or three nerve crushes muscle weight and strength, when expressed as per cent of values found in unoperated controls, differed only slightly. Also, muscle tension elicited by nerve stimulation, expressed as per cent of that elicited by direct stimulation, varied only slightly whether the nerve had been crushed once, twice or three times. Thus the degree of change which took place during this essentially degenerative period was of about the same magnitude after repeated denervations as after a single denervation. These findings mean that the amount of atrophy which followed any denervation was related to the amount of losable weight (muscle cell phase) present in the muscle at the time of any one denervation and was unaffected by the occurrence of previous denervations.

The data obtained in studies made at 42 days after terminal denervation afforded an opportunity for evaluating the changes which took place during a period in which reinnervation and regeneration were predominant. The studies showed that the weight and strength of regenerating muscle were slightly greater after two or three denervations than after a single denervation. The tension developed in re-

TABLE 1. SUMMARY OF THE EFFECT OF REPEATED DENERVATION ON THE STRENGTH AND WEIGHT OF THE GASTROCNEMIUS MUSCLE OF THE RAT

	NO. OF CRUSHES	TIME ¹	NO. OF ANIMALS	TENSION PER GRAM MUSCLE CELL PHASE WHEN ACTIVATED THROUGH				RELATIVE WT. MUSCLE CELL PHASE	$\frac{\text{TENSION (NERVE}^2\text{)}}{\text{TENSION (MUSCLE}^2\text{)}} \times 100$
				Nerve		Muscle			
				grams	per cent ²	grams	per cent ²		
Exper.	1	21	10	1452	69.0	2105	97.5	51.0	68.4
Control	0	—		2123		2159			98.2
Exper.	2	21	9	1092	55.0	1984	95.0	49.0	55.0
Control	0	—		1984		2087			95.0
Exper.	3	21	9	1438	58.6	2333	90.6	47.0	61.7
Control	0	—		2453		2574			95.0
Exper.	1	42	10	1630	75.8	2278	99.0	76.0	71.7
Control	0	—		2150		2300			93.4
Exper.	2	42	10	2085	93.3	2395	108.4	88.0	88.3
Control	0	—		2234		2209			101.5
Exper.	3	42	10	2082	87.6	2294	92.0	85.0	90.7
Control	0	—		2375		2491			95.4
Exper.	1	84	10	2295	100.4	2408	102.3	90.0	95.2
Control	0	—		2286		2352			97.2
Exper.	2	84	10	2302	91.8	2324	95.8	93.0	98.2
Control	0	—		2397		2424			98.8
Exper.	2	21	9	1092	97.7	1977	102.3	52.0	55.2
Control	1	21	10	1116		1932			57.7
Exper.	3	21	9	1438	129.0	2241	116.0	55.0	64.3
Control	1	21	10	1116		1932			57.7
Exper.	2	42	10	2085	120.0	2358	117.8	90.0	88.5
Control	1	42	10	1741		2004			87.0
Exper.	3	42	10	2082	84.7	2394	119.5	87.0	87.0
Control	1	42	9	2457		2004			86.2
Exper.	2	84	10	2302	107.3	2314	95.3	94.0	99.3
Control	1	84	10	2139		2431			88.0

¹ After last denervation.

² Expressed as percentage of that found in contralateral control.

³ Stimulation.

sponse to nerve stimulation, in per cent of tension developed in response to direct stimulation, was slightly higher in the double and triple denervations than after single denervations.

At the 84th day, when regeneration was nearly complete, comparisons of the effects of two denervations with a single denervation showed that tension and weight values and the ratios of tension elicited through nerve stimulation to that through direct stimulation were essentially of the same order in both groups. The volume of

reinnervation at this time was essentially normal in both the single and repeated denervation groups.

The findings described above were also apparent in the experiments in which the controls for the repeated lesions consisted of singly denervated muscles. These findings as a whole show that skeletal muscle can be subjected to repeated denervation by nerve crushing without significantly altering the course of subsequent reinnervation and regeneration. The accumulative effects of repeated denervations were slight and in the direction of more effective reinnervation and regeneration.

It is of interest to speculate as to whether comparable results would have been obtained if, instead of denervation by nerve crushing, some other procedure such as section and suture had been employed. The latter method would have offered a better opportunity for reinnervation through axon branching but less favorable opportunities because of a greater scar tissue barrier. The method of denervation employed in these studies was the one which offered the promise of the most consistent responses in terms of quantitative atrophy and regeneration.

SUMMARY

The left tibial nerves of 120 female albino rats were crushed once, twice or three times, at intervals of 42 days. Strength and wet weight of the muscles supplied by these nerves were measured at 21, 42 and 84 days after the terminal lesion and compared with values obtained from unoperated control muscles. Comparisons were also made between muscles whose nerves had been crushed twice or three times and those whose nerves had been crushed only once.

Measurements of weight changes (calculated on the basis of muscle cell phase changes), tension per gram of contractile tissue (elicited by both indirect and direct stimulation) and tension by nerve stimulation in per cent of tension by direct stimulation show that *a*) during the predominantly degenerative phase (21 days after the last lesion) values from one, two and three crushes showed no significant variation; *b*) during the predominantly regenerative phase (42 days) values were slightly higher after two and three crushes than after one, and *c*) during the phase of nearly complete recovery (84 days) values after two crushes were very nearly the same as after a single crush.

These results indicate that skeletal muscle can undergo repeated cycles of denervation atrophy and recovery without being significantly impaired in its regenerative capacity. The accumulative effects of such procedures are of small magnitude and point in the direction of a more effective reinnervation and recovery during the regenerative phase.

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ROLE OF ACETYLCHOLINE IN THE ACTIVITY OF SENSORI-MOTOR AND SUPPRESSOR AREAS OF THE CORTEX¹

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THAT acetylcholine (ACh) with or without eserine exerts an excitatory effect on cortical activity has been shown by the study of cortical potentials (1-3), and by the observation that excitatory motor phenomena follow the application of eserine or ACh to the motor cortex (4, 5). Since no experiments seem to have been reported in the literature in which the rôle of ACh for the cortical suppressor areas has been investigated this action was studied. In addition, some experiments on the effect of cholinesterase (ChE) on cortical potentials are likewise reported.

METHOD

The experiments were performed in dial-urethane anesthesia mostly on cats but a few rabbits and three monkeys (macacque) were used also. In the exposure of the lateral surface of the brain and in the bipolar recording of the potentials the technique was the same as in previous papers from this laboratory.

Eserine sulfate (1%), ACh chloride (10%), Acetyl-beta-methyl choline (Merck) (2.5-10%) and ChE (1-2%) were used. The above solutions were diluted in mammalian Ringer or saline and the pH adjusted to 6.6. Controls were run with mammalian Ringer or saline adjusted to the same pH.

The ChE was prepared from dog pancreas following the method described by Mendel and Mundell (6), with minor modifications and omitting precipitation with ammonium sulfate at 0.5 saturation and succeeding steps. The activity of the ChE was determined at room temperature (24°C.) by electrometric titration with a Coleman pH meter using 1 cc. of a 1 per cent acetylcholine and 0.1 cc. of a 0.1 per cent ChE solution. It was found that 1 mgm. of ChE hydrolyzed 0.225 mgm. ACh per minute.

In addition to the *in vitro* studies ChE was also tested on biological assay preparations. It prevented the usual responses of the rectus abdominis muscle and the Straub heart preparation to ACh and this effect was abolished after heating (cf. table 1).

The effect of ACh and eserine or of ChE on normal brain activity was investigated by applying the substance being studied to the cortex either on strips of filter paper 1 x 4 mm. placed between the electrodes or on discs about 4 mm. in diameter with small sectors cut out for the electrodes. The effect of filter paper dipped in saline served as control.

The action of ACh and eserine on suppressor areas was studied by means of electrocorticograms and electromyograms, the latter elicited by stimulation of the

Received for publication February 11, 1948.

¹ Aided by a grant from the Office of Naval Research.

motor cortex. The lateral suppressor areas were identified on the basis of the maps of Garol (5) for the cat and of Dusser de Barenne and McCulloch (7) for the monkey. *Area 24* or the corresponding region in the cat was first tested electrically, stimulation resulting in pupillary dilation and/or suppression of motor activity. Electromyograms were generally recorded from the following hindleg muscles: semimembranosus and tendinosus, gastrocnemius and tibialis. When electromyograms were recorded the motor cortex was stimulated bipolarly at constant intervals with condenser dis-

TABLE 1.

RESPONSE OF THE RECTUS ABDOMINIS (FROG) TO ACETYLCHOLINE BEFORE AND AFTER TREATMENT WITH CHOLINESTERASE

<i>Test Solution</i>	<i>Height of Contraction</i>
1. ACh 1:660,000	7.5 mm.
2. ACh 1:660,000	7.5 mm.
3. ChE 1:5,000 for 5 min., then ACh 1:660,000	3.5 mm.
4. ACh 1:660,000	5.0 mm.
5. ACh 1:660,000	8.0 mm.

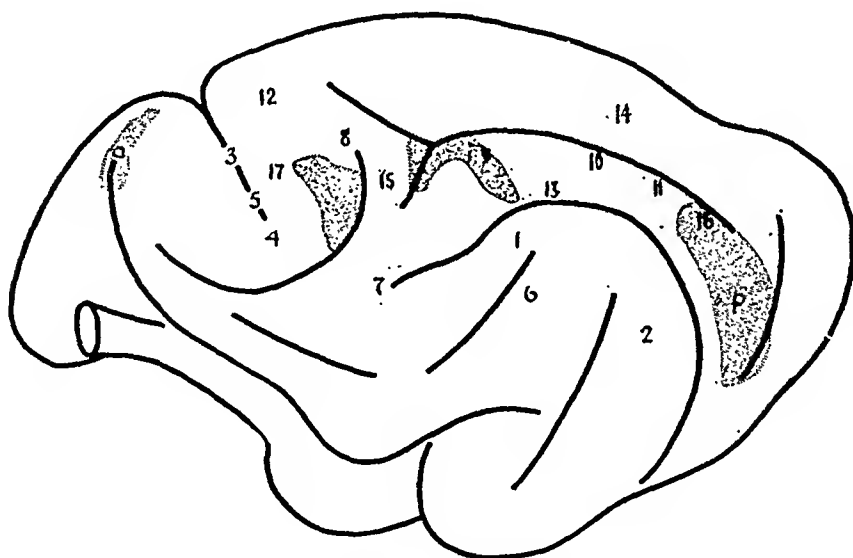


Fig. 1. CEREBRAL CORTEX OF THE CAT (5). Numbers refer to the sites mentioned at the left side of the records or in the legends to the figures; *i*, intermediate, *p*, posterior suppressor area.

charges, frequency 90, duration 10 sigma, for five seconds. The cortical sites used in this study are indicated in figure 1 for the cat.

1. *Rôle of ACh in the activity of the suppressor areas.* Although failure to stimulate the suppressor areas was not uncommon even with electrical stimuli, data were obtained showing that ACh and eserine were effective stimulants. In these experiments the action of the suppressor areas was demonstrable as a decline in or complete suppression of cortical potentials, cortically induced movements or spontaneous movements such as tremor.

Figure 2 illustrates suppression of cortical activity at sites 10² and 2 on the

² cf. figure 1 for the sites stimulated.

right cortex. Control activity was recorded for 13 minutes. Application of saline-soaked filter papers to both right intermediate and posterior suppressor areas (*i* and *p* of fig. 1) and their removal in no way altered the cortical potentials (A). Then one per cent eserine was applied to the same sites and caused no change in cortical potentials (B). However, after it had been replaced by 10 per cent ACh complete suppression of R 10 and partial suppression of R 2 occurred (C and D). Three minutes after ACh application R 10 exhibited partial recovery (E) lasting for three minutes. This stage was followed by suppression of both sites for 10 minutes (F). Removal of ACh led to recovery of the potentials (G) which was not complete until 20 minutes later.

On the same animal a second suppression experiment was attempted by using the left suppressor areas and recording potentials from *sites 10* and *11* of that side (fig. 3). This experiment was unique among several suppression experiments in that suppression followed the application of eserine without succeeding administration of ACh. Spontaneous cortical activity recorded for 10-minute periods was the same before, during and after the application of saline to the left intermediate and posterior suppressor areas (A). One per cent eserine was applied to both suppressor areas and induced at first no changes in the E. C. G. (B). Several minutes later suppression of cortical activity began (C) and increased (D, E). Inasmuch as the suppression differed in onset and duration for the two sites, the possibility that these changes are due to circulatory disturbances can be ruled out.

Eserine and ACh applied to the left intermediate and posterior suppressor areas of another cat caused bilateral suppression of cortical activity. Complete suppression lasted for five and one-half minutes on the right side and seven and one half minutes on the left. Recovery was complete five minutes after the ACh was removed and the brain washed.

One experiment on a monkey indicated that the leg region of 2s when excited by eserine plus ACh suppressed activity only in the leg region of *areas 1* and *4* (fig. 4). The electrocorticogram was recorded for 45 minutes prior to the test and remained unaltered after saline was applied to the leg region of 2s and after its removal (A). One per cent eserine alone had no effect (B), but about one minute after it was replaced by 10 per cent ACh, spontaneous activity was suppressed in the leg areas (C). *Sile III* (arm region of *area 4*)³ remained unchanged while *I* and *II* (leg region) showed the greatest suppression (D). Progressive degrees of recovery are recorded in E to G. They are noteworthy for two reasons: *a*) record F shows more 'dial' potentials than were seen under control conditions; *b*) record G taken several minutes later shows large frequent potentials while 'dial' groups are completely absent. These data suggest that during the recovery from cortically induced suppressions the cortex passes through a stage of deeper anesthesia to one of relative excitation. The latter seems to be analogous to the 'rebound' of cortical potentials commonly seen on recovery from anoxia and asphyxia (8).

The effect of excitation of the medial suppressor area with eserine and ACh on cortically induced movements may be seen in figure 5. E. M. G.'s indicated that the muscle responses to stimulation of the motor cortex under control condition.

³ The activity in the arm region of *area 1* which is not included in figure 4 was likewise unaltered.

(A) and after application of eserine (B) were constant. C taken after 10 per cent ACh had been applied to the suppressor area showed complete suppression of the previous slightly responding hamstring muscles and almost complete suppression of tibial activity. The onset of response of the tibialis is much delayed when compared with the onset of responses *a* and *b*. After removal of ACh recovery occurred (D).

In another cat evidence was obtained for suppression of a spontaneous tremor as well as of cortically induced movements. This suppression (fig. 6) occurred when the application of eserine to the medial suppressor area followed that of mecholyl. Mecholyl alone was ineffective on spontaneous activity as well as on responses of muscles to cortical stimuli (A, A'). B and B' show that eserine had no effect at first, but later spontaneous activity ceased (C') and the response to cortical stimulation decreased greatly (C). Ten minutes after eserine had been applied there was a slight recovery of brain function indicated by the reappearance of some spontaneous activity (D') and greater response to cortical stimulation (D). After eserine had been removed complete recovery occurred (E and E').

II. *ChE and spontaneous cortical activity.* ChE applied to various parts of the cerebral cortex resulted in diminished activity characterized by potentials of decreased amplitude and frequency. Figure 7 illustrates this effect on the cortex of a monkey. Similar records were obtained on rabbits. All effects were reversible.

In experiments performed on cats the control activity consisted primarily of groups of 'dial' potentials. Under these conditions ChE diminished the amplitude of the 'dial' bursts without altering the frequency. More rarely the spontaneous activity was completely eliminated.

The characteristic effect of ChE usually occurred 2 to 12 minutes after the initial application. Sometimes ChE and control filter papers were resoaked in their original solutions. Occasionally this replenishment was necessary to alter cortical activity with ChE. In other observations the second application started a second depression after partial or complete recovery from a previous one.

Potentials from other cortical sites serving as controls (cf. fig. 7) were always recorded simultaneously to eliminate the possibility of a decline in brain activity due to circulatory changes. Such changes would occur simultaneously in all regions. The diminution in cortical potentials produced by ChE seldom lasted more than four minutes. When the period of decline was short, recovery usually occurred while the ChE was still in place; in other experiments recovery generally followed ChE removal and washing of the cortex within three minutes.

III. *ACh and cortical responsiveness to afferent stimuli.* That ACh plays a rôle in the responsiveness of the cortex to afferent stimuli was shown in a series of experiments in which the effect of stimulation of the sciatic nerve on cortical potentials was studied after ACh, eserine or ChE had been applied to one or more cortical

Fig. 2. SUPPRESSION OF E.C.G. FOLLOWING APPLICATION OF ESERINE (Es) AND ACETYLCHOLINE (Ac) TO LATERAL SUPPRESSOR AREAS. Cat: 2nd experimental day. Horizontal line, 5 sec. Calibration, 150 microvolts. A. Control (8 min. after saline removed). B. 5½ min. after 1% Es on intermediate and posterior suppressor areas. C. ½ min. after 10 per cent Ac on suppressor areas. D, E, F and G. 2.5, 4.5, 8.5 and 24.5 min. after Ac, respectively. H. 45 min. after Ac washed off.

Fig. 3. SUPPRESSION OF E.C.G. FOLLOWING APPLICATION OF Es TO LATERAL SUPPRESSOR AREAS, SECOND EXPERIMENTAL DAY. Horizontal line, 5 sec. Calibration, 150 microvolts. A. Control (5 min. after saline was removed.) B, C, D, E, F and G. One, 8.5, 10.5, 13 and 16.5 min. after Es.

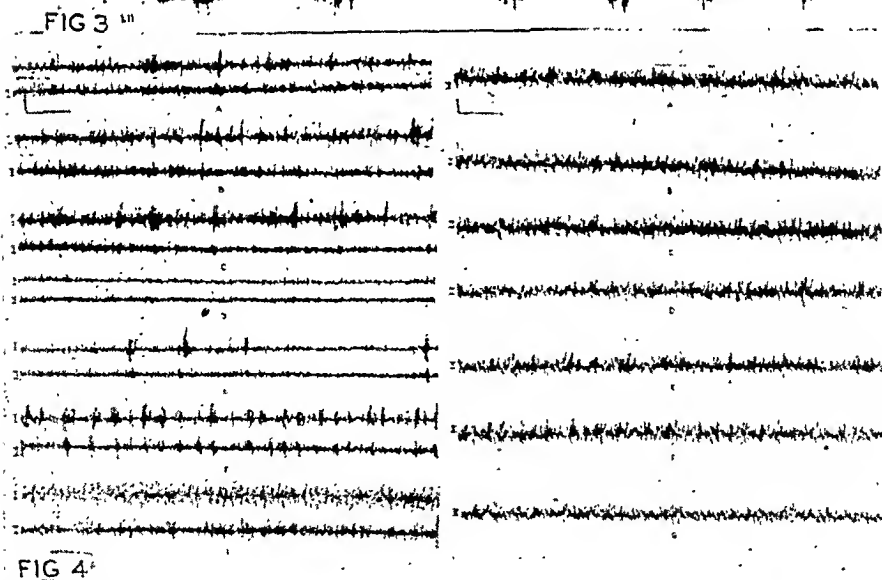
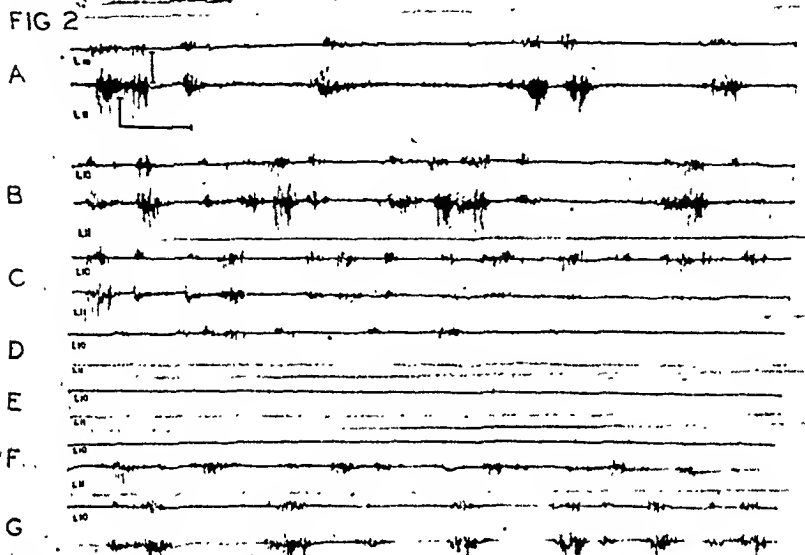
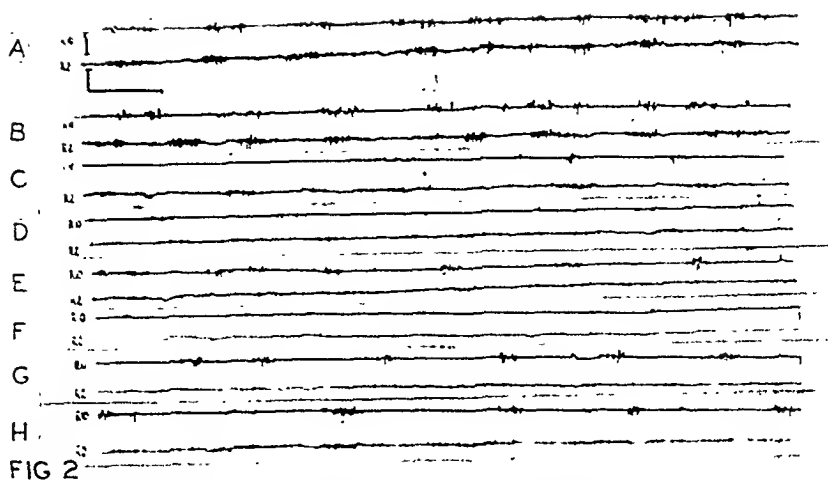


Fig. 4. SUPPRESSION OF E.C.G. OF LEG REGION OF AREAS I AND 4 FOLLOWING APPLICATION OF ES AND AC TO LEG REGION OF AREA 2S. *Monkey*: I, leg region of area 4; II, leg region of area 1; III, arm region of area 4. (right half of fig. 4). Horizontal line, 5 sec. Calibration, 100 microvolts. A. Control (4 min. after saline removed). B. 9 min. after 1% Es on leg region of area 2s. C. 1 min. 45 sec. after 10% Ac on leg region of 2s. D, E and F. 2.5, 4, and 6.5 min. after Ac. G. 2.5 min. after Ac washed off and 13 min. after Ac applied.

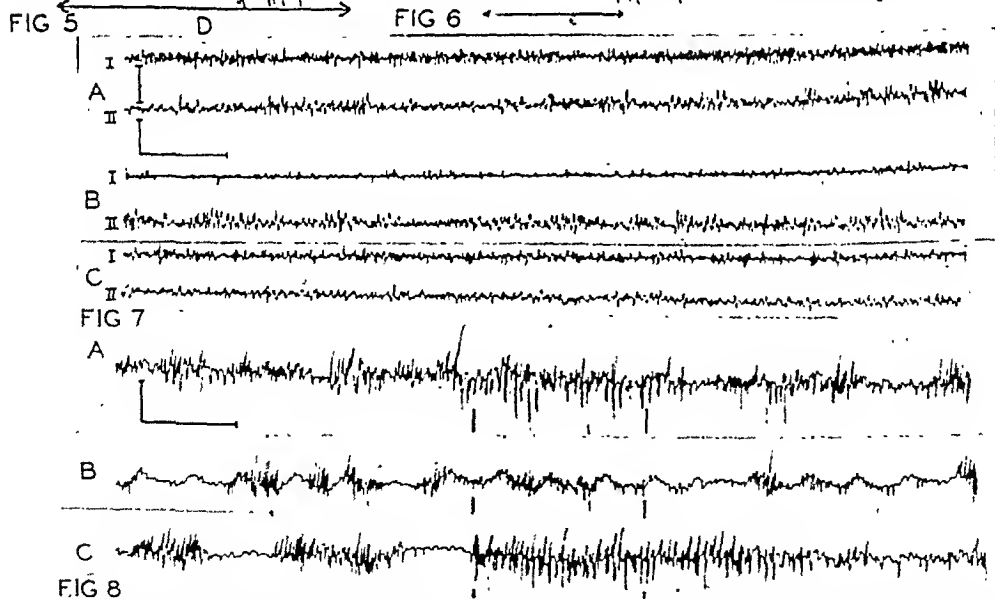
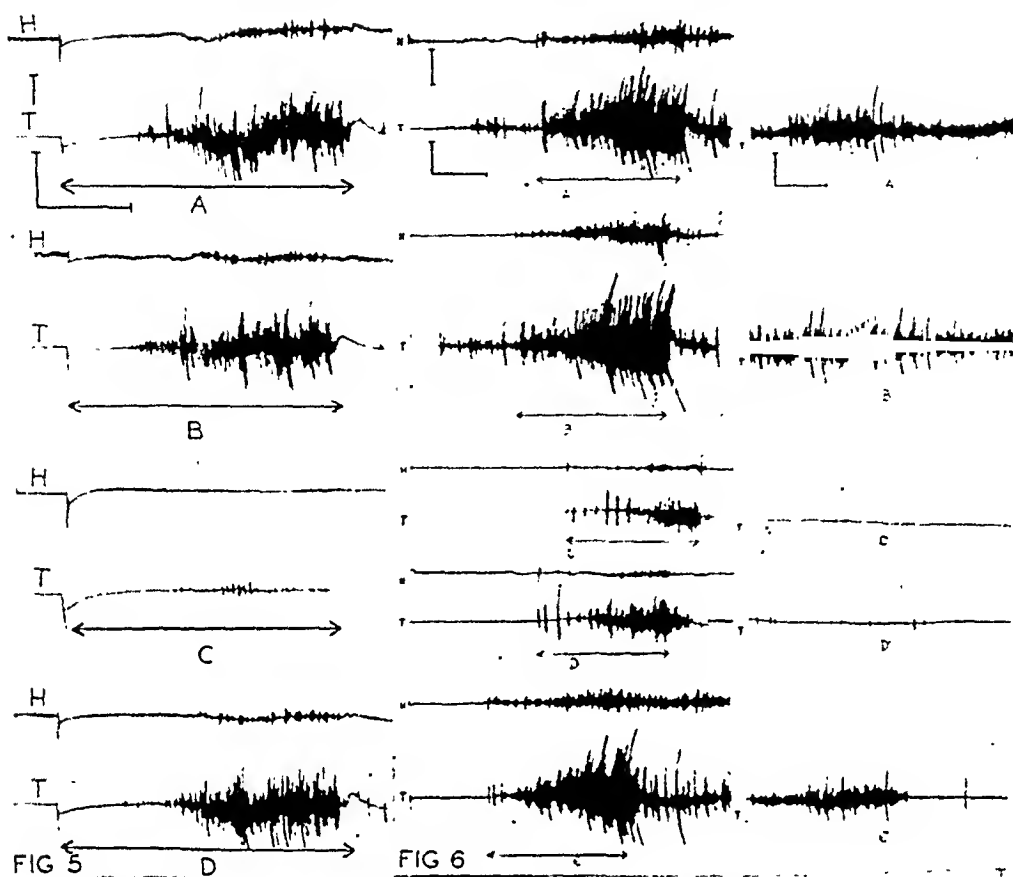


Fig. 5. SUPPRESSION OF E.M.G.'s FOLLOWING APPLICATION OF ES AND AC TO MEDIAL SUPPRESSOR AREA. *Cat*: left cortex removed. E.M.G.'s from left semimembranosus and tendinosus muscles (H) and left tibialis (T). Horizontal line, 2 sec. Calibration, 100 microvolts. Right motor cortex every 3 min. with condenser discharges for 5 sec. between arrows with 4.8 volts, 90/sec. A. Control. B. 8 min. after 1% Es on medial suppressor area. C. 2 min. after 10 per cent Ac on medial suppressor area. D. 2.25 min. after Ac removed and cortex washed.

Fig. 6. SUPPRESSION OF SPONTANEOUS AND CORTICALLY INDUCED E.M.G.'s BY APPLICATION OF ES FOLLOWING ACETYL-BETA-METHYL-CHOLINE TO MEDIAL SUPPRESSOR AREA. *Cat*: 2nd experimental

sites while other sites treated with saline served as controls. It was found that ACh, eserine or the combination of both increased the response of the cortex to sciatic stimulation, the type of response being similar to that previously described (9). On the other hand pretreatment of a cortical site with ChE diminished or abolished the response to sciatic stimulation. Figure 8 shows such an effect in spite of the fact that the resting cortical activity was altered but slightly by topical application of ChE. Whereas the E. C. G. showed large frequent potentials during the stimulation of the sciatic nerve prior to the application of ChE (A) the response after application of ChE consisted of potentials of very low amplitude and increased frequency. This effect was reversible (C). In the light of earlier work (11) this type of reaction seems to indicate that ChE diminishes the action of afferent impulses on the cortex although this action as well as that on spontaneous activity was not always demonstrable.

DISCUSSION

The experiments have clearly established the fact that the lateral and medial suppressor areas of cat and monkey can be stimulated by local application of eserine plus ACh. Such stimulation calls forth the two forms of cortically induced suppressions (6) which result in a diminution of cortical potentials and/or in a suppression of movements induced by electrical stimulation of the motor cortex. The spontaneous activity not infrequently seen in recording E. M. G.'s of lightly anesthetized monkeys is likewise suppressed. All effects are reversible.

Several authors mentioned in the introduction noted that eserine plus ACh excite cortical neurons. Our own experiments have shown that the medial and lateral suppressor areas may likewise be excited by ACh or mecholyl particularly under the stabilizing influence of eserine. Evidence has also been obtained showing that changes in the cortical potentials which result from stimulation of the sciatic are more marked after topical application of eserine plus ACh than in preceding and following control experiments. On the other hand our experiments have demonstrated that topical pretreatment of a cortical site with ChE reduces or abolishes spontaneous cortical activity in cat and monkey (confirming Tokaji's observation on the brain of the frog), (12) and diminishes the effect of sciatic stimulation on the E. C. G.

day, both suppressor areas exposed. E.M.G.'s from right semimembranosus and tendinosus muscles (H) and right tibialis (T). Calibration, 50 microvolts. Left horizontal line, 2 sec.; right, 4 sec. Left motor cortex with 4.0 volt condenser discharges for 5 sec. every 2½ min. (except when substances were applied or removed at which time a longer interval occurred between stimuli). Right-hand column labeled A'-E' is spontaneous activity just preceding each stimulation. In previous experiment 2½% acetyl-beta-methyl-choline applied to both suppressor areas. A. Control. B. 2½ min. after one per cent Es on right and left medial suppressor areas. C. and D. six and 10 min., respectively, after Es. E. 7½ min. after Es washed off.

FIG. 7. EFFECT OF CHOLINESTERASE ON SPONTANEOUS CORTICAL ACTIVITY. Monkey: I., E.C.G. from arm region area 1; II, E.C.G. from leg region area 1. Horizontal line, 5 sec. Calibration, 150 microvolts. A. Control. B. 2 min. after cholinesterase 1:100 applied to I, saline to II. C. Recovery 45 min. after B.

Fig. 8. EFFECT OF CHOLINESTERASE ON THE CORTICAL RESPONSE (SITE 11) TO SCIATIC STIMULATION IN THE CAT. Stimulation of the sciatic (11 V, 90/sec., for 10 seconds) between vertical lines A. Control. B. 11' 15" after application of cholinesterase 1:100. C. 16' 15" after application and 11' 30" after removal of cholinesterase.

These data warrant the statement that the activity of sensori-motor and suppressor neurons may be increased by ACh or factors stabilizing this substance (eserine) and decreased by factors increasing its rate of destruction (ChE).

The observation that ACh with eserine may induce excitation of motor as well as suppressor neurons of the cortex in a manner similar to that induced by electrical stimulation suggests that ACh is an important link in the chain of chemical events associated with excitation of cortical neurons. The investigations of Abdon (13, 14) showing that a precursor of ACh breaks down in conjunction with the activity of heart and skeletal muscle and that these processes are independent of stimulation of nerves seem to support such an interpretation.

No attempt has yet been made in the experiments reported in this paper and in the investigations of Miller, Chatfield, Merritt and their collaborators (2-4) to determine whether or not in the reactions studied synaptic transmission is essentially altered.

SUMMARY

The topical application of acetylcholine, eserine and cholinesterase prepared from the pancreas of dogs exerts the following effects on the cortex of the brain in cats and monkeys: 1. Eserine with acetylcholine stimulates suppressor areas as indicated by temporary suppression or diminution of cortical electrical activity and by temporary elimination of the responsiveness of the motor cortex to electrical stimulation as recorded by electromyograms. These substances also increase the responsiveness of the cortex to afferent stimuli as indicated by electrocorticograms. 2. Cholinesterase diminishes cortical activity and the responsiveness to afferent stimuli.

It is inferred from these observations and the data reported in the literature that acetylcholine is linked up with the process of excitation in motor, sensory and suppressor neurons on the cortex.

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EFFECT OF AGE ON LETHALITY OF DI-ISOPROPYL FLUOROPHOSPHATE

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THE rôle of acetylcholine in the central nervous system has as yet not been clearly established, although it is widely held that this substance participates in the propagation of the impulse in the peripheral nervous system. Due to the small quantities of acetylcholine present in the tissues and the great rapidity of its hydrolysis, direct study is well-nigh impossible (1). As has proved the case in analogous situations, the methods of enzyme chemistry have been helpful in the solution of this problem. The activity of acetylcholine is intimately bound up with the enzyme that hydrolyzes it, namely cholinesterase. Depression of cholinesterase permits accumulation of acetylcholine. On the other hand, increase in the activity of cholinesterase decreases acetylcholine. Since cholinesterase is a stable enzyme and can be determined readily by reliable methods, investigation of this enzyme yields significant information concerning acetylcholine.

In the past decade, study in this manner of the acetylcholine-cholinesterase relationship has advanced our knowledge of nervous mechanisms. Yet there is no unanimity of opinion, particularly in regard to the central nervous system. Some investigators in this field hold that the acetylcholine-cholinesterase relationship is crucial and indispensable for normal functioning of the brain; others believe that it is without such significance (2, 3). The discovery of the action of di-isopropyl fluorophosphate (DFP), a toxic compound which either irreversibly inhibits or destroys cholinesterase (4), opened new pathways for the investigation of nervous mechanism both in the peripheral and central nervous system. Aside from its anticholinesterase effect, DFP has no action that has been conclusively demonstrated. This anticholinesterase activity, however, is powerful and specific and is, therefore, an important tool for the study of acetylcholine.

If two animal populations could be found that differed markedly in the cholinesterase concentration of the brain, the presence or absence of a variation in response to DFP might be indicative of the relative importance of this brain enzyme and its substrate, acetylcholine. Since Nachmansohn (5) has shown that the brain cholinesterase concentration of the adult rat is more than four times that of a newborn and Welshe and Hyde (6) have also demonstrated that the brain acetylcholine content of the adult rat is four times that of a newborn, our two populations were available.

The experiments were conducted in two parts: first the relative susceptibility of rats of various ages to DFP was studied; secondly, the mechanism of the difference was investigated.

Received for publication February 24, 1948.

METHODS

DFP was weighed out in amounts of 200 to 300 mgm. and placed in hard glass ampules. Ampules were crushed in known volumes of propylene glycol. The propylene glycol solution was then diluted with appropriate amounts of normal saline yielding solutions requiring the injection of 0.1 to 0.5 cc. per animal.

Stock white rats of both sexes and of various ages were used in these experiments, the adults weighing up to 200 grams. Injections were made subcutaneously and intraperitoneally. In the case of the very young rats, arterial clamps were used to seal the needle puncture in order to prevent loss of even small amounts of fluid. For controls, newborn rats were injected with the vehicle used for the DFP.

To obtain information on the part played by the brain in the reaction to DFP, brain cholinesterase was determined on newborn and adult rats 30 minutes following the subcutaneous injection of 2.0 mgm/kgm. DFP. The method was the manometric technic using the Warburg respirometer (5, 7). A remarkable lowering of cholinesterase activity was found with this amount of DFP, so that the usual concentration

TABLE 1. DFP RETENTION IN RAT BRAIN

	ANTICIPATED VALUE	ACTUAL VALUE	INHIBITION
			%
Adult			
1	376	361	4
2	194	185	5.4
3	196	201	—
Newborn			
1	39	38	2.6
2	198	200	—
3	103	143	—
4	143	129	10

of brain homogenate introduced into the respiratory chamber in dealing with the normal rat was inadequate to produce measurable quantities of CO_2 . Using varying amounts of the same brain homogenate it was observed that 250 to 400 mgm. of brain were necessary in each respiratory chamber. When such large amounts of brain are used in each flask the CO_2 and acid retention by protein becomes very important (8). The acid retention determined in the manner described by Umbreit (8) and Dixon (9) was found to alter the flask constant significantly both in the adult and in the newborn. On the other hand, CO_2 retention (8) was not of importance. Thus, in all determinations of DFP-poisoned animals protein retention was taken into account.

Another possible correction to be considered is the DFP which might be retained in the brain tissue, and therefore might lower the actual cholinesterase activity during grinding. The DFP retention was checked in the manner described by Bullock *et al.* (10), but no significant retention was disclosed (table 1).

Lastly, the brain cholinesterase in adult rats was determined 30 minutes following the subcutaneous injections of varying amounts of DFP. Clinical signs were carefully observed.

RESULTS

The results of the subcutaneous injection of 175 rats are summarized in figure 1. In this diagram five-hours' survival are charted in order to avoid confusion with secondary effects such as inanition, but the results on a 48-hour basis are essentially similar. First, it may be noted that all the newborn controls survived; second, that all injected newborns succumbed; third, with advance of age a generally increasing resistance up to a 73 per cent survival in the adult rats occurred.

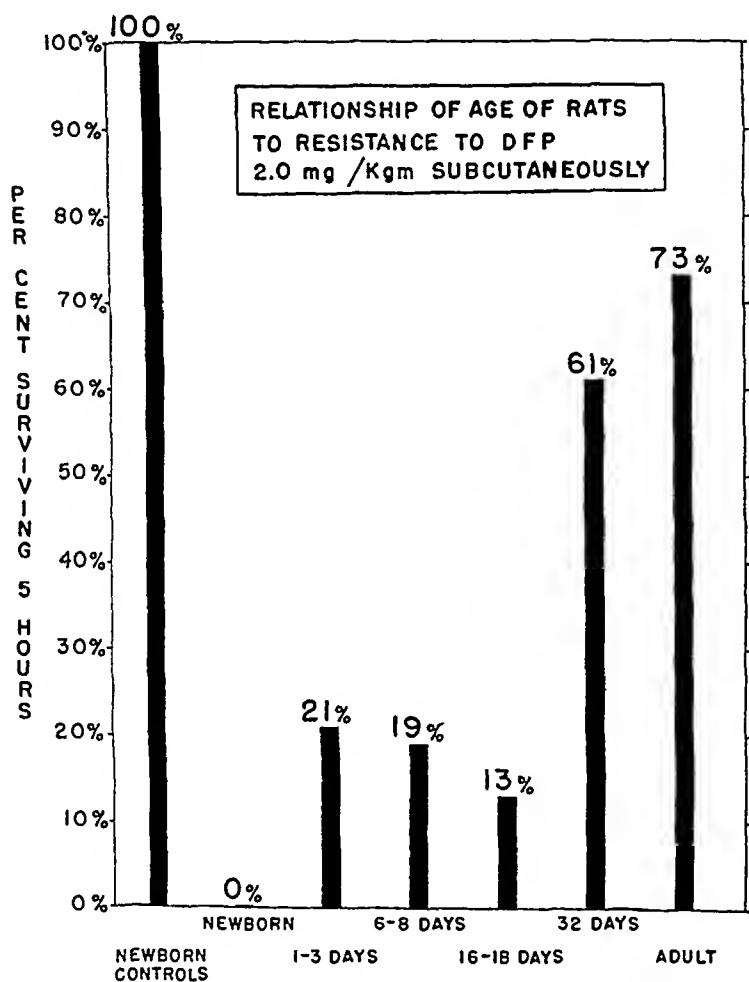


Fig. 1

The results of the intraperitoneal injections are, in general, in keeping with those obtained by subcutaneous injection.

Table 2 indicates brain cholinesterase activity in the adult and newborn normal rat and again after DFP. One is immediately struck by the marked lowering of brain cholinesterase activity by DFP—a decrease from 917 to 27 cu. mm. $\text{CO}_2/100$ mgm. tissue/hr. in the adult and from 186 to 8 in the infant. Although the absolute amount of activity is significantly greater in the adult than the infant, the per cent

remaining is approximately the same—3 per cent in the adult and 4 per cent in the newborn.

The correlation between increasing severity of clinical signs and diminution of brain cholinesterase activity is brought out in table 3. The correlation observed in the adult rat is so consistent that it can scarcely be considered to be fortuitous. Furthermore, it appears that the critical level of brain cholinesterase activity permitting survival in the adult lies between 16 and 39 cu. mm. CO₂/100 mgm. tissue/hr.

TABLE 2. BRAIN CHOLINESTERASE CO₂ CU MM/100 MGM. BRAIN/HR. DFP 2 MGM/KGM SUBCUTANEOUS

	CONTROL	DFP	REMAINING ACTIVITY
			%
Adult	917 ± 168.6	27.3 ± 5.96	3
Newborn	186 ± 45.12	8.1 ± 3.96	4

TABLE 3. RELATIONSHIP BETWEEN CLINICAL SIGNS OF DFP TOXICITY AND BRAIN CHOLINESTERASE ACTIVITY

NO.	DFP MGM/KGM. SUBCUT.	CLINICAL SIGNS	BRAIN CHOLINESTERASE CO ₂ /100 MGM./BRAIN/HR.
6	0	None	917
3	0.5	Increased mouth movements, exophthalmos.	256-409
7	1.0	Slight trembling, fasciculation, moderate weakness of the hind limbs, some hyper-reactivity on tapping spine, 'champing'.	60-178
8	2.0	Marked salivation, 'champing', pronounced weakness of all limbs, inability to crawl, exaggerated trembling, marked hyper-reactivity on tapping spine.	20-39
6	3.0	Above signs in more severe form, tonic and clonic convulsions, either in extremis or dead.	11-16

It is of interest to note that the signs observed in the newborn poisoned with 2 mgm/kgm. DFP were at first 'champing', then increased motion of the limbs, worm-like movements of the body, cyanosis, cessation of respiratory movements and, eventually, death.

DISCUSSION

In order to explain the marked difference in susceptibility to DFP between the adult and newborn several possibilities must be entertained. First, one may consider the possibility of different rates of absorption from the subcutaneous tissue of the adult and the newborn. However, the essential similarities in the results using either the subcutaneous or the intraperitoneal route is evidence against any such expla-

nation. Secondly, one may hold that the adult might be able to deviate and retain more DFP in the lipoid substance in the cerebral white matter which is much more abundant in the adult. However, investigation of the DFP retention has shown that such is not the case (table 1). By the same token, the failure to demonstrate significant DFP retention in the newborn brain indicates that the detoxification apparatus of the newborn is adequate for this dose of DFP and that the inability to destroy DFP does not explain the higher mortality in the newborn. Third, it is possible that the greater resistance of the adult is associated with the higher level of cholinesterase activities in the mature brain. The adult initially presents a higher level of cholinesterase activity than the newborn. Both groups suffer a per cent depression of equal amount after the injection of DFP. However, the adult is left with a higher absolute cholinesterase activity because of its higher starting value. Thus the adult rat retains an amount which in most cases appears sufficient to sustain life. On the other hand, the newborn beginning with a less intense activity is left with a lower value. It is not known if the crucial level of brain cholinesterase activity is identical for the adult and the newborn. If one assumes they are approximately the same, then the longer survival in the adult is related to its higher level of brain cholinesterase. The possibility remains, however, that the infant requires a lesser activity than the adult to sustain life, a possibility that can be decided only by further work. This great sensitivity of the newborn is in striking contrast to its greater resistance in other conditions such as anoxia (11), hypoglycemia (12), morphine poisoning (13) and alcohol toxicity (14). However, this is consistent with its greater sensitivity to barbiturates (15).

The greater vulnerability of the newborn rat to an anticholinesterase has many implications. It is well known that human infants are much more prone to convulsive episodes than adults. Since it has been suggested by many that convulsions are frequently associated with an excess of acetylcholine (16, 17), this tendency of the young toward convulsions may be due to their greater sensitivity to various anticholinesterases both physiological and pathological. In the latter regard, one may recall that Gesell (18) has shown that CO_2 is a most potent anticholinesterase.

Finally, we may discuss the rôle of the acetylcholine-cholinesterase relationship in the central nervous system. Investigators in this field have long regarded changes in cholinesterase as an indicator of reciprocal changes in acetylcholine; thus a diminution of cholinesterase activity is accompanied for a period of time by an excessive accumulation of acetylcholine. Further, the symptoms of acute DFP toxicity have been considered to be those of acetylcholine accumulation. Since it has been suggested in these experiments that survival in the presence of an anticholinesterase is correlated with the maintenance of a high level of cholinesterase in the brain, these findings add further evidence that preservation of the balance in the acetylcholine-cholinesterase relationship is crucial for the normal functioning of the brain.

SUMMARY

1. Newborn rats are much more sensitive to DFP than adults. During the growth period, resistance to DFP increases with age until the rat is about 120 days old. The impaired resistance of the newborn has been tentatively explained on the

basis of lower cholinesterase concentration in the newborn brain, and consequently a lower reserve or safety factor.

2. A correlation between clinical signs and level of brain cholinesterase has been observed. The more severe symptoms are observed with greater depression of cholinesterase level.

3. These experiments point to a critical rôle for acetylcholine and cholinesterase in the central nervous system.

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ON THE ANTITHROMBIC AND ANTIPROTEOLYTIC ACTIVITY OF ALPHA TOCOPHERYL PHOSPHATE^{1, 2, 3}

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DURING the course of investigations on respiration in muscle large doses of alpha tocopheryl phosphate (α -TPh) were administered to rats and to man. Profound effects on a variety of biological processes were observed; among these, alterations in the normal process of coagulation of blood were especially striking.

There are indications from the observations of several investigators that modifications of blood coagulation may accompany disturbances in the economy of vitamin E. Thrombosis was discovered in fetal rats dying in mothers which were vitamin E deficient (1). Hyaline thrombi in capillaries in and about degenerated areas of the cerebellum were believed to be the primary lesions in the encephalomalacia of chicks produced by a diet deficient in vitamin E (2, 3). Furthermore, the chemical structure of α -tocopherol provides a potential biological antagonist to vitamin K, and indeed, vitamin K deficiency has followed the administration of α -tocopheryl quinone, a derivative produced by mild oxidation (4). Finally, naphthotocopherol, a related compound, exhibits some of the biological activity both of vitamin E and of vitamin K (5).

This communication reports a direct measurement of the effects of α -TPh on the clotting of blood. Because certain phases of coagulation of blood may be simulated by proteolysis (6) and inhibited by antiproteases (7-10), the influence of α -TPh on a series of representative proteases was measured.

METHODS

The effect of α -TPh was observed on the following systems *in vitro*: a) coagulation time of recalcified human plasma, b) thrombin clotting time of prothrombin-free human plasma, c) digestion of casein by plasma protease, trypsin, leucoprotease and papain. The effect *in vivo* was studied by measurement of thrombin clotting time of plasma following parenteral administration of α -TPh to rats. The disodium salt of d,l- α -tocopheryl phosphate⁴ was used throughout these studies because its solu-

Received for publication March 17, 1948.

¹ Work performed under a contract between the Office of Naval Research, Navy Department and the Johns Hopkins University.

² Assisted by a grant-in-aid from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry, American Medical Association.

³ Presented to the American Federation for Clinical Research, New York, N. Y., December 12, 1947.

⁴ Drs. Elmer Sevringhaus and Leo Pirk of Hoffmann-LaRoche, Inc., Nutley, New Jersey, generously supplied the α -tocopheryl phosphate.

bility in water makes it suitable for use both in *in vitro* systems and for parenteral administration. The pH of an aqueous solution of one per cent α -TPh is 8.14, determined in the Beckman pH meter. For use *in vitro* in the study of blood coagulation, all solutions of α -TPh were freshly prepared in 0.85 per cent sodium chloride. For parenteral administration, aqueous solutions of α -TPh were used. For the experiments on proteolysis, α -TPh was dissolved in Sørensen $\frac{M}{15}$ phosphate buffer, pH 7.4.

Plasma was prepared immediately before use from human blood to which was added 0.1 M sodium oxalate in a ratio of 0.5 ml. to 4.0 ml. of whole blood.

Prothrombin-free plasma was prepared by shaking fresh human oxalated plasma with an excess of barium sulfate and centrifuging. The supernate was proven prothrombin-free by recalcification and testing with thromboplastin by a modified Quick method (11).

The thrombin employed was a commercial preparation of bovine thrombin (Parke, Davis). For the *in vitro* studies thrombin was dissolved in 0.85 per cent sodium chloride in concentrations which would yield clotting times of 15 to 30 seconds in control systems. In the experiments performed on blood drawn from rats following parenteral administration of α -TPh, there was insufficient plasma to titrate the thrombin solutions so that the thrombin clotting time of the control blood did not always fall within these limits.

In the latter experiments, varying doses of α -TPh, as indicated in table 3, were injected intraperitoneally into adult Whelan rats under sodium pentobarbital anesthesia and blood withdrawn under direct observation from the right ventricle 30 minutes later into a syringe containing 0.1 M sodium oxalate, usually in a ratio of 0.5 ml. for each 4.5 ml. of whole blood. While it was not always possible to obtain such a ratio, the final ratio of oxalate to whole blood was always constant throughout each experiment. Thrombin clotting times in this instance were measured by addition of a solution of bovine thrombin to oxalated rat plasma which had not been rendered prothrombin free.

Details of the coagulation systems employed are provided in the accompanying tables.

The progress of proteolysis was measured nephelometrically at a wave length of 405 m μ with a Coleman Junior spectrophotometer (12). In two series of experiments, concomitantly with nephelometry, proteolysis was followed by determination of nitrogen appearing in the protein-free supernate of the reaction systems by a modified Koch-McMeekin method (13).

The concentrations of plasma protease preparation (0.02 ml./ml.) and of leucoprotease preparation (0.02 ml./ml.) were selected to cause approximately 50 per cent digestion of casein in 20 hours. Crystalline trypsin⁵ (0.00025 mgm./ml. = 7×10^{-9} M) and crude papain (Difco, 0.01 mgm./ml.) caused nearly complete proteolysis of casein in 20 hours. Plasma protease was prepared by the method of Tagnon (14). The leucoprotease preparation was an homogenate in 15 volumes of distilled water of cat polymorphonuclear leucocytes derived from sterile serous exudates (12).

RESULTS

When α -TPh was added to human plasma in a final concentration of 1.8×10^{-3} M, clotting time after recalcification was prolonged, as indicated in the representative experiment in table 1. Concentrations one-tenth this amount, $1.8 \times$

⁵ Kinly provided by Dr. M. Kunitz, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

10^{-4} M, were associated with a slight acceleration of clotting time which was of doubtful significance. It has been demonstrated that α -TPh forms an insoluble monocalcium salt (15). It is evident from table 1, however, that an equimolar combination of calcium with α -TPh (1.8×10^{-3} M) could not have removed sufficient calcium to prevent clotting, since the final molar concentration of calcium was three to 13 times that of α -TPh.

TABLE 1. EFFECT OF ALPHA-TOCOPHERYL PHOSPHATE IN VITRO ON COAGULATION OF RECALCIFIED PLASMA

Oxalated Plasma	1.8×10^{-2} M α -TPh	0.85% NaCl	9×10^{-2} M CaCl_2	Coagulation Time
ml.	ml.	ml.	ml.	min.
0.5	—	0.9	0.1	9
0.5	—	0.8	0.2	6
0.5	—	0.7	0.3	7
0.5	—	0.6	0.4	7.3
0.5	0.15	0.75	0.1	> 20
0.5	0.15	0.65	0.2	> 20
0.5	0.15	0.55	0.3	> 20
0.5	0.15	0.45	0.4	> 20

Human plasma, α -TPh and sodium chloride were added in the order listed to pyrex glass test tubes and incubated at 37°C . for three minutes before addition of calcium chloride. All tubes were set up in duplicate. The first series was examined for coagulation at 30-second intervals. When complete coagulation occurred in a tube of the first series, the duplicate tube was then examined every 30 seconds. Clotting time is recorded as that time at which complete coagulation was detected in the duplicate tube.

TABLE 2. EFFECT OF ALPHA-TOCOPHERYL PHOSPHATE IN VITRO ON THROMBIN-FIBRINOGEN SYSTEM

α -TPh	Clotting Time	α -TPh	Clotting Time
M	seconds	M	seconds
0	17.0	2×10^{-4}	34.2
2×10^{-5}	25.8	2×10^{-3}	> 120.0

The coagulation system consisted of 0.2 ml. oxalated prothrombin-free human plasma, 0.2 ml. of varying concentrations of α -TPh in 0.85 per cent sodium chloride and 0.1 ml. of an aqueous solution of crystalline bovine thrombin. Plasma and tocopherol were incubated for two minutes in pyrex glass test tubes at 37°C . before addition of thrombin. Clotting time was that time, after addition of thrombin, at which coagulation first could be detected. All values are averages of four to six determinations.

In an effort to determine the site of inhibition of clotting, the effect of α -TPh on the thrombin-fibrinogen system was studied. To prothrombin-free human plasma were added varying quantities of α -TPh and a solution of bovine thrombin, as illustrated by table 2. As little as 2×10^{-5} M of α -TPh prolonged the clotting time of the test solution, indicating that α -TPh is vigorously antithrombic.

When α -TPh was injected intraperitoneally into rats⁶ and blood withdrawn 30 minutes later the thrombin clotting time was prolonged by doses of 0.05 gram per kgm. of body weight and larger, as shown in table 3. A dose of 0.01 gram α -TPh per kgm. was ineffective. Mixtures of incoagulable plasma from an α -TPh-injected rat

⁶ When α -TPh was injected into normal rats, a single large dose, one or two grams per kilogram of body weight, subcutaneously or intraperitoneally, uniformly produced apparent drowsiness, a taxia and profound weakness, occasionally convulsions and death in the course of several hours.

and normal rat plasma could not be coagulated by the addition of an amount of thrombin which was adequate to produce coagulation in normal rat plasma alone.

The effect of α -TPh was determined on the proteolytic activity of plasma protease, crystalline trypsin, leucoprotease and papain. These proteolytic enzymes were inhibited markedly by α -TPh except in the case of papain, which was inhibited only slightly (table 4). Crystalline trypsin, 7×10^{-9} M, was inhibited completely by concentrations of α -TPh as low as 5×10^{-4} M, but incompletely inhibited by lesser concentrations. It should be noted, however, that the preparation of crystalline trypsin used in these experiments contains 50 per cent magnesium sulfate. The final concentration of magnesium sulfate in the system illustrated in table 4 was at least 2×10^{-6} M. We have observed that a precipitate was formed when a solution of magnesium sulfate or chloride was added to a solution of α -TPh, and it has been

TABLE 3. EFFECT OF ALPHA-TOCOPHERYL PHOSPHATE IN VIVO ON COAGULATION OF RAT PLASMA BY THROMBIN

Exp. No.	Rat No.	α -TPh gram/kilo	Clotting Time seconds
1	1	0	25.6
	2	2	53.0
2	3	0	10.0
	4	2	14.2
	5	2	22.7
3	6	0	9.5
	7	0.1	15.6
	8	0.1	23.9
4	9	0	23.2
	10	0.05	120.0
5	11	0	18.5
	12	0.01	18.9

The coagulation system consisted of 0.2 ml. of oxalated plasma from rats injected with varying amounts of α -TPh and 0.1 ml. of an aqueous solution of crystalline bovine thrombin. The reaction proceeded at 37°C.

assumed that this precipitate is an insoluble magnesium- α -TPh, analogous to the insoluble calcium salt of α -TPh reported by others (15). In the trypsin system the concentration of magnesium was sufficient to remove at least a portion of the α -TPh from solution and, presumably, from participation in the enzyme system.

Since the assumption was made that magnesium and α -TPh formed an insoluble complex, it was necessary to determine whether or not inhibition by α -TPh of tryptic digestion was the result of removal of magnesium ions from the enzyme system. Accordingly, the proteolysis of casein by the crystalline trypsin preparation was measured in the presence of an excess (10^{-4} M) of sodium carbonate and of sodium citrate. Precipitation of magnesium carbonate and of magnesium citrate from the reaction systems had no effect on proteolysis, suggesting that magnesium ions were not essential to tryptic digestion and that the inhibition exerted by α -TPh was not the result of loss of magnesium from solution.

TABLE 4. INHIBITION OF PROTEOLYSIS BY ALPHA-TOCOPHERYL PHOSPHATE

Enzyme	α -TPh M	Inhibition per cent
Papain 0.5 mgm./100 ml.	0	0
	10^{-4}	0
	5×10^{-4}	33
	2×10^{-3}	100
Plasma Protease	0	0
	10^{-4}	31
	5×10^{-4}	100
	2×10^{-3}	100
Leucoprotease	0	0
	10^{-4}	100
	8×10^{-4}	100
Crystalline Trypsin 7×10^{-9} M	0	0
	10^{-6}	7
	10^{-5}	10
	10^{-4}	21
	5×10^{-4}	100
	2×10^{-3}	100

The test system consisted of 3.5 ml. of 0.5 per cent casein, 0.1 ml. or 0.2 ml. of enzyme, 0.1 ml. to 1.0 ml. of varying concentrations of α -TPh in phosphate buffer, pH 7.40, and sufficient Sørensen $\frac{M}{15}$ phosphate buffer, pH 7.40, to make a final volume of 10.0 ml. The reactants were combined serially in the following order: substrate, α -TPh and enzyme. The reaction proceeded at 37°C. The presence of at least 2×10^{-6} M of magnesium sulfate in the trypsin system resulted in precipitation of an undetermined moiety of α -TPh. The effective concentration of α -TPh was therefore less than that stated in the table.

DISCUSSION

The experimental data presented above indicate that α -TPh is vigorously antithrombic and antiproteolytic.

The average concentration of tocopherols in normal human serum is one mgm. per cent, a concentration of the order of 2×10^{-5} M (16). From table 2 it will be recalled that this concentration proved to be detectably antithrombic. This similarity, when viewed in the light of the thromboses which occur in experimental vitamin E deficiency (1-3), suggests the possibility that tocopherols participate normally in maintaining that equilibrium which prevents intravascular coagulation of blood.

There are insufficient data to define the mechanisms by which α -TPh exerts its antithrombic and antiproteolytic activity. Although certain reducing agents are antithrombic (17) and antiproteolytic against trypsin, leucoprotease and plasma protease (18), it is unlikely that these effects of α -TPh are in simple consequence of the antioxidant property of tocopherols in general. α -TPh is not an antioxidant; it exhibited neither appreciable reducing capacity for sodium 2,6-dichlorobenzenone-indophenol nor any reduction potential in plasma or in the proteolytic systems examined in this study.

It is possible that α -TPh is reduced reversibly *in vivo* and that its antithrombic effect *in vivo* depends upon its ability to function as a reducing agent. Such an

explanation demands the unsatisfactory proposition that the antithrombic effect of α -TPh *in vivo* is the result of a process quite different from that responsible for its effect *in vitro* on thrombin.

The possibility has not been eliminated that parenteral administration of α -TPh stimulated the release of heparin. The fact that α -TPh was potently antithrombic *in vitro*, however, suggests that this activity is independent of heparin.

The structural relationship between α -tocopherol and naphthoquinone has been emphasized by others (4, 5), and the effects of some naphthoquinones are similar to certain effects of α -TPh; both groups of compounds inhibit the succinoxidase system (19, 20, 21) and both inhibit thrombin activity (17). The explanation of the phenomena described in the present report may lie in an, as yet, undefined property endowed by some structural configuration shared by the tocopherols and certain naphthoquinones.

SUMMARY

1. Disodium d,l- α -tocopheryl phosphate (α -TPh), a water-soluble derivative of α -tocopherol, prolongs the clotting time of recalcified plasma. This effect is not the result of precipitation of ionic calcium from the system.

2. α -TPh is antithrombic, both *in vitro* and when injected intraperitoneally into rats. Its effect *in vitro* is exerted at concentrations of the order of that of tocopherol in normal human plasma.

3. α -TPh is antiproteolytic against plasma protease, crystalline trypsin, leuco-protease, and to a slight degree, papain.

Mrs. Marjorie Glass and Mrs. Martha Jaffe contributed valuable technical assistance. Dr. C. L. Conley generously gave advice in the coagulation studies.

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INFLUENCE OF THE DOSAGE OF XANTHOPTERIN UPON THE RESPONSE IN HEMAPOIESIS

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XANTHOPTERIN has been shown to give a response in red blood cell (RBC) regeneration of anemic fish (1) and anemic rats (2). The response in both cases was proportional to the dosage of xanthopterin.

Xanthopterin has also been shown to accelerate cell proliferation in *in vitro* cultures of bone marrow from various animals (3). With increasing concentrations of xanthopterin the cell proliferation reaches a maximum at approximately 5 γ per ml. of bone marrow suspension. With concentrations below 5 γ per ml. there is an increase in cell proliferation with increasing concentration and with concentrations of xanthopterin above 5 γ per ml. there is a rapid decrease in the rate of cell proliferation.

Thinking that there might be a similar maximum or optimum response with xanthopterin when injected into anemic animals, high levels of xanthopterin were injected and the results are given in the present paper.

In the first experiment with increased dosage, young rats which had been made anemic on a purified diet¹ containing one per cent sulfathiazole were injected with 24 mgm. of xanthopterin per kilogram body weight. At the time of injection the average blood values of the group were: RBC 5.7×10^6 per cmm.; WBC 3960 per cmm.; hemoglobin 10.5 grams per 100 ml. and cell volume 35.6 per cent. On the third day after injection all the rats of the group died, but analysis was made of their tail blood before death. The results obtained on the third day after injection with 24 mgm. of xanthopterin per kilogram body weight were: RBC 2.1×10^6 per cmm.; WBC 1490 per cmm.; hemoglobin 4.5 grams per 100 ml.; and cell volume 14.3 per cent.

In 1941 Horlein (4) investigated the toxicity of xanthopterin and leucopterin. When injected intravenously as the sodium salt the lethal dose was found to be 50 mgm. per kilogram for mice; 30 mgm. per kilogram for rabbits and 7.5 mgm. per kilogram for cats. Death did not follow immediately but occurred several days later. Necrosis of the pancreas and kidney damage were observed but no report was made of blood cell or hemoglobin findings.

As our above experiment indicated marked changes in blood values, further tests were made.

Received for publication March 8, 1948.

¹ The diet used was composed of: glucose 72 per cent, casein 18 per cent, cottonseed oil 3 per cent, cod liver oil 2 per cent, salt mixture 4 per cent and sulfathiazole 1 per cent. The following vitamins were added per kilogram of diet: thiamine-HCl 10 mgm., riboflavin 20 mgm., pyridoxine 10 mgm., Ca-pantothenate 40 mgm., niacin 20 mgm., 2-methyl, 1-4 naphtho-quinone 4 mgm., biotin 0.01 mgm. and choline-Cl 2 grams.

METHODS AND RESULTS

Rats about five months old, which had been made anemic on a purified diet¹ containing one per cent sulfathiazole were divided into groups of from 4 to 7 rats each. The rats of each group were given a single intraperitoneal injection of the dosage indicated and the subsequent blood changes observed are given in figure 1. The groups given a single injection of one mgm. and 5 mgm. per kilogram body weight showed an increase in blood values, reaching a maximum in approximately 12 days after injection followed by a rapid decline toward the initial value. The groups given the single injection of 10 and 20 mgm. per kilogram, respectively, showed a decrease in the blood values measured. Although the anemia was aggravated by

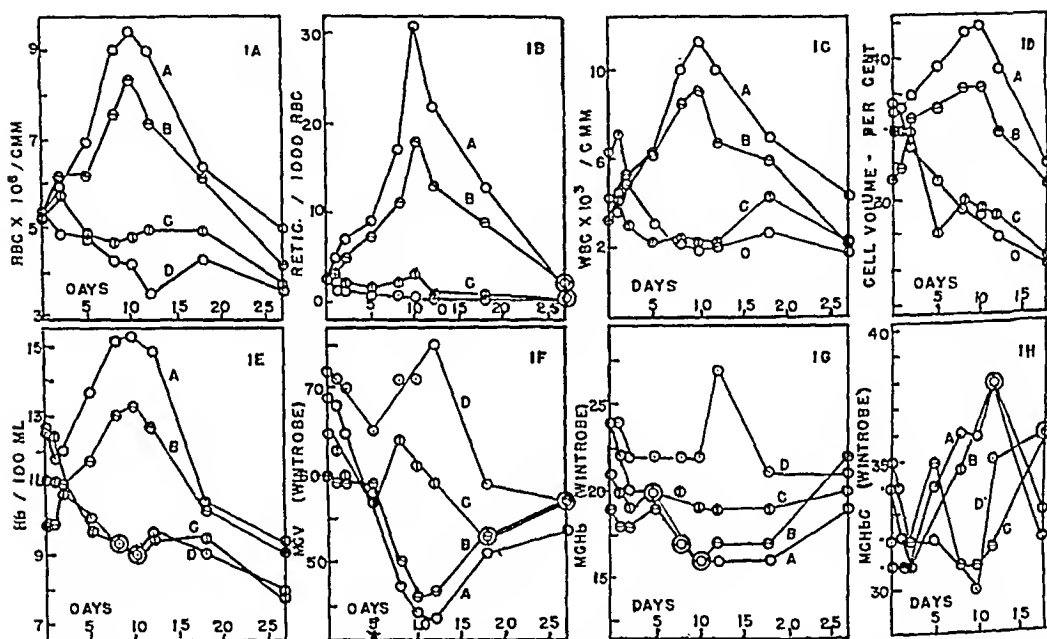


Fig. 1. EFFECT OF A SINGLE INJECTION of different amounts of xanthopterin on the blood values of nutritionally anemic rats. The curves represent the response with dosages of xanthopterin as mgm. per kilogram body weight of: A, 1; B, 5; C, 10; and D, 20.

the excessive dosage of xanthopterin all of the rats survived. Figure 1 also gives the calculated values for the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCHb) and mean corpuscular hemoglobin concentration (MCHbC) according to Wintrobe. Before injection with xanthopterin all of the animals appeared to exhibit a macrocytic anemia. The MCV for the groups injected with one and 5 mgm. per kilogram dropped to within the range of values observed for normal rats for the period from approximately the sixth to the eighteenth day after injection, corresponding with the period of increased cell count. The MCV for the groups injected with 10 and 20 mgm. per kilogram remained above the values on normal rats.

After conclusion of the above experiment the rats which had been used in it were divided into two groups as nearly equal as possible in weight and past history with regard to previous dosage with xanthopterin. The average initial blood values for

each group were very similar as indicated in the graph. The rats of one group were injected intraperitoneally with one mgm. of xanthopterin per kilogram and of the other group with 3.18 mgm. of synthetic pteroylglutamic acid (folic acid) per kilogram, which would give a pteroyl concentration similar to that of the xanthopterin used. The second injection was made 31 days after the first injection. Figure 2 shows the response obtained. After the effects of either an optimal dosage of xanthopterin or a toxic overdosage had worn off, the second injection of one mgm. per kilogram of xanthopterin gave a response very similar to the first. The pteroylglutamic acid produced but slight response.

Xanthopterin had such a marked effect upon the blood picture of nutritionally anemic rats it was thought possible that it might have some effect upon the normal animal. Rats ranging from 165 to 217 grams, with an average weight of 182 grams and on a diet of fox checkers, were divided into six groups and given a single injection of xanthopterin. Each animal was injected intraperitoneally with 2.5 ml. of

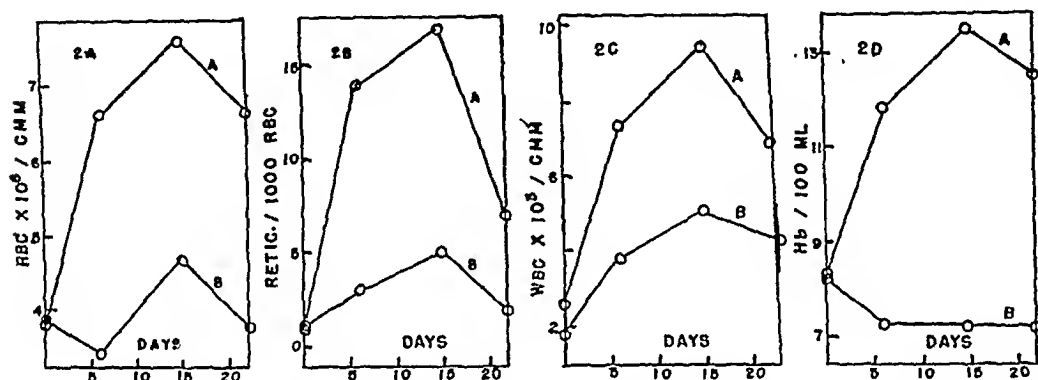


Fig. 2. EFFECT OF A SINGLE INJECTION of one mgm. per kilogram of xanthopterin (Curve A) and of 3.18 mgm. per kilogram of folic acid (Curve B) upon the blood values of nutritionally anemic rats which had recovered from the effects of a previous injection of xanthopterin.

NaHCO₃ solution containing the supplements for the six groups as follows: none, 2.5, 5, 10, 25 and 50 mgm. per kilogram. The tail blood of each rat was analyzed at intervals and averages for the groups are given in figure 3. Figure 3 also gives the Wintrobe indices calculated from the data obtained. The results on 5, 10, 25 and 50 mgm. per kilogram show the production of a macrocytic anemia with a leukopenia, while 2.5 mgm. per kilogram appeared to produce a polycythemia with leukocytosis.

To further study what appeared to be a polycythemia with the lower concentration of xanthopterin, another experiment was performed using younger rats. Rats about two months old, which had been raised on a diet of fox checkers, were divided into groups of four rats each. The rats were given a single injection of 2.5 ml. of NaHCO₃ solution containing the supplements as follows; none, 1, 2, 5 and 50 mgm. of xanthopterin per kilogram. The results obtained are given in figure 4. These animals were not mature and the values for RBC of the control group follow very closely the average values given by Kindred and Corey (5) for rats of the same age.

The group injected with one mgm. per kilogram showed a polycythemia and leukocytosis. The RBC increased over two million cells per cmm. within nine days

after injection to a value of 10.2×10^6 per cmm. The WBC increased from about 8000 to 19,000 per cmm. in two days and to 22,000 by the ninth day after injection. The groups injected with 2 and 5 mgm. per kilogram had an increase in blood values

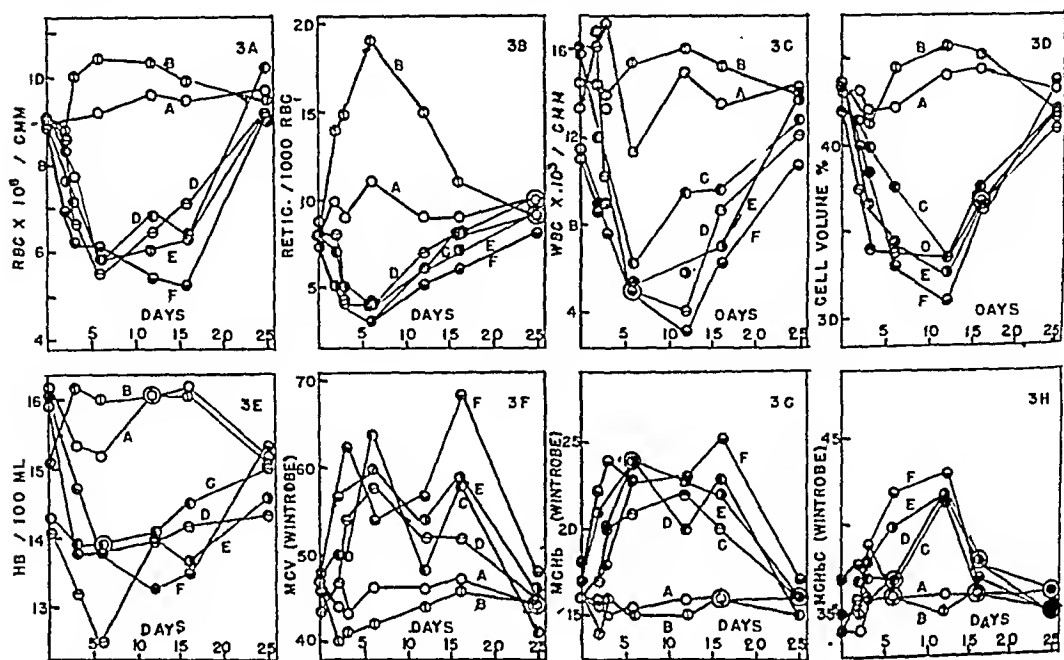


Fig. 3. EFFECT OF A SINGLE INJECTION of different amounts of xanthopterin on the blood values of normal rats. The curves represent the results with dosages of xanthopterin as mgm. per kilogram body weight: A, none; B, 2.5; C, 5; D, 10; E, 25; F, 50.

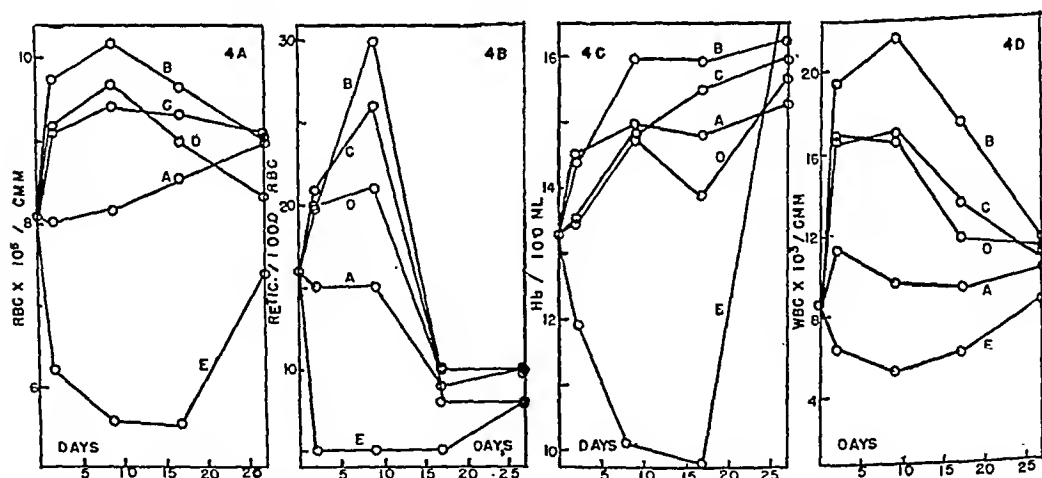


Fig. 4. EFFECT OF A SINGLE INJECTION of different amounts of xanthopterin on the blood values of normal rats. The curves represent the results with dosages of xanthopterin as mgm. per kilogram body weight of: A, none; B, 1; C, 2; D, 5; and E, 50.

but less marked than for one mgm. per kilogram. Five mgm. per kilogram produced an anemia in the previous experiment, reported above, and a polycythemia in this experiment in which younger rats were used. The group injected with 50 mgm. per

kilogram developed an anemia with a drop of 2.5×10^6 RBC per cmm. within nine days. All values approached normal after about 30 days from the time of injection.

Spies (6) reported that he did not get a 'response' in a pernicious anemia case upon injection of 500 mgm. of xanthopterin each day for 10 days, a total of 5000 mgm. of xanthopterin. He does not give the weight of his patient but the amount administered must have been appreciably greater than any of the dosages used here when calculated to mgm. per kilogram body weight.

SUMMARY

1. Dosages of less than 5 mgm. of xanthopterin per kilogram body weight produce hemapoiesis in nutritionally anemic rats with an apparent optimum of one mgm. per kilogram.

2. Dosages of 10 mgm. or more of xanthopterin per kilogram body weight aggravate the condition in nutritionally anemic rats, making the animals more anemic.

3. Dosages of less than 5 mgm. of xanthopterin per kilogram body weight produce polycythemia and leukocytosis in normal rats, with one mgm. per kilogram giving the highest values observed.

4. Dosages of greater than 5 mgm. of xanthopterin per kilogram body weight produce anemia and leukopenia in normal rats. The anemia has a tendency to be of a macrocytic type.

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RESIDUAL LUNG VOLUME DETERMINATIONS BY THE METHODS OF HELIUM SUBSTITUTION AND VOLUME EXPANSION¹

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THE measurement of air remaining in the lungs after forced expiration has been attempted by various methods including direct measurement on the cadaver, indirect measurement by water displacement of the extirpated lungs, and by calculation of the nitrogen content of the lungs *in vivo*. Herein reported are 63 determinations of residual lung volume made on six trained subjects, five of whom were deep sea divers.

METHODS

Three methods were employed: *a*) dilution of residual air with oxygen, *b*) substitution of residual air with helium-oxygen mixture and subsequent measurement of helium and *c*) measurement of gas volume resulting from expansion of residual lung volume during a rapid reduction of air pressure from four to one atmospheres in a naval recompression chamber.

Nitrogen Dilution Method. Initial determinations were made in the general manner described by Van Slyke and Binger (1) in which the nitrogen contained in residual air was diluted by rebreathing a known volume of pure oxygen until complete mixing was attained between the gas in the lungs and the oxygen in the rebreathing system. In later tests forced breathing was employed. Four liters of oxygen were introduced into a system containing two rubber bags and a mask arranged so that the gas in the two bags could be mixed. The subject exhaled to the residual air volume and then rebreathed deeply into the bag system 12 times per minute for one and one-half minutes. The subject again exhaled to the residual air volume. The mask tubing was clamped to permit mixing and sampling of gases in the two bags. The residual air space was computed by use of the following formula (2):

$$RA = \frac{V(N_2 - a) + V_{m_2} N_2 - V_{m_1} a}{79.1 - N_2}$$

Where V_{m_1} is the volume of the bags measured before the test; V_{m_2} is the volume at

Received for publication January 7, 1948.

¹A résumé of tests conducted at the Experimental Diving Unit, U. S. Naval Gun Factory Washington, D. C., October 1941.

the end of the test; V is the volume of the dead space; N_2 is the percentage of nitrogen in the bag system at the end of the test; and α is the percentage of nitrogen at the start of the test.

Helium Substitution Method. The measurement of residual air by the helium substitution method was devised to eliminate a source of error attendant upon the nitrogen dilution method, namely, measurement of nitrogen in an atmosphere chiefly composed of nitrogen where a slight leak precludes accuracy.

The residual air of the lungs was replaced by a known helium-oxygen mixture. The helium was then washed out of the lungs by the inhalation of air or oxygen and its volume measured.

The equipment for collecting helium from the residual lung volume consisted of two 100-liter rubber bags cross-connected by three-quarter inch rubber tubing and equipped with a face mask, a two-way valve, check valves, tubing clamps and an open circuit helium-oxygen supply to the mask alone (fig. 1).

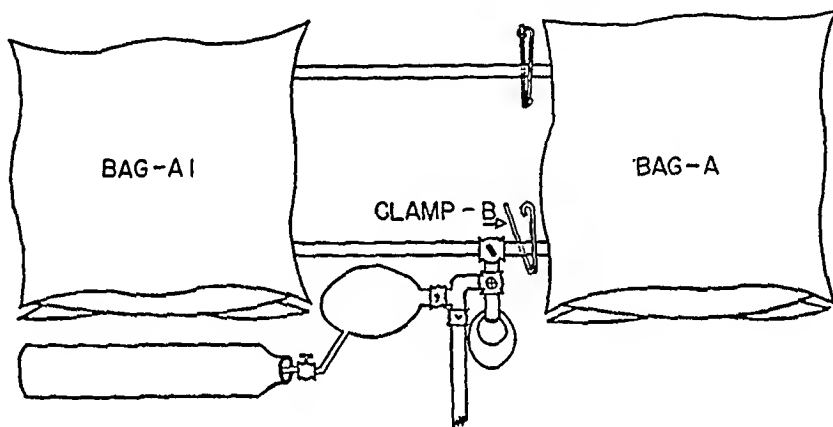


Fig. 1. DIAGRAM OF APPARATUS used for measuring residual lung volume by helium dilution.

The seated subject inhaled a 79 per cent helium-21 per cent oxygen mixture and maintained maximal deep breathing for a period of three minutes. Breathing was timed with a stop watch and regulated to provide 5-second inspiration and expiration at the rate of six respirations per minute. Following the final maximal expiration to residual lung volume, the two-way valve was turned so that 50 liters of oxygen previously measured into bag A were breathed from bag A to bag A¹; the same respiratory pattern was continued. In order to prevent a free flow of gas between the two bags due to pressure difference, hand-manipulated clamp B was opened during inspiration and closed during expiration. After emptying bag A, which required about two minutes, the subject again exhaled to residual lung volume and the two-way valve was closed. The clamps were then removed to permit to-and-fro movement of the gas within the bags, and a sample of mixed gas was analyzed for helium in the Cady apparatus, in which all gases in the mixture except helium were adsorbed by activated charcoal at the temperature of liquid air. That rinsing the lungs with 50 liters of gas was adequate to remove the helium was shown by tests in which only 2 to 11 cc. of helium could be removed after two minutes of hyperventilation (25L/min.).

CALCULATIONS. The volume of helium recovered from the lungs during the two-minute washing-out period was computed by multiplying the volume of the oxygen-helium system by the percentage of helium found on analysis. The residual lung volume was computed from the following formula:

$$V_R = \frac{V_B \left(1 - \frac{W_B}{760} \right) \frac{He_B}{100}}{\left(1 - \frac{W_L}{760} \right) \frac{He_I}{100}} - V_D$$

When V_R = residual vol., V_B = bag vol., V_D = dead space, He_B = per cent He in bag, He_I = percentage He inspired, W_W and W_L = partial pressure of water vapor in bag and lungs, respectively. No correction was made for the effect of changes in the respiratory quotient on alveolar helium percentage.

Volume Expansion Method. The equipment used consisted of a 10-liter rubber bag attached to a face mask by a short three-quarter inch rubber tube closed by a hand clamp. By means of a recompression chamber, the barometric pressure was raised to four atmospheres absolute. The apparatus, having been exhausted of air at normal barometric pressure, was tightly fitted to the face following complete forced expiration. The hand clamp was removed from the tubing and the chamber rapidly vented from four to one atmospheres during a period of about 25 seconds. The subject meanwhile exhaled into the bag as the residual air expanded. Upon reaching normal atmospheric pressure, complete forced expiration was made again and the tubing clamped. The gas contained in the bag was the result of the physical expansion of residual lung volume which occurred during the decompression from four to one atmospheres, plus the gas contained in the dead space of the mask. The residual air volume was computed from the following formula:

$$RV = \frac{V_b - \frac{B - 760}{760} (V_D)}{\frac{B - 760}{760}}$$

Where V_b is the bag volume and V_D is the volume of the dead space. Results were corrected to 760 mm. and to 37° C.

A pressure of less than four atmospheres can be used, but with decreased accuracy. It is not necessary to vent the chamber in the 25-second period as outlined. A slower reduction of pressure is possible if correction is made for oxygen consumption during the time period. A pressure reduction greater than four to one atmospheres was not used due to the possible formation of air emboli during the rapid decompression. The absence of symptoms of air emboli indicates that the method employed was safe. However, fatigue followed repeated daily determinations.

Sources of Error. The chief reason for inconsistency of results was the inability of subjects to exhale to the same residual air volume during successive trials. For accuracy it was necessary to give the subjects a number of practice runs. In the calculations underlying the helium substitution and pressure-reduction methods some error is introduced by assuming that the ratio of carbon dioxide output to oxy-

gen consumption was unity. In the pressure-reduction method a small error may be introduced by the assumptions that the gas in the bag at the end of the test was saturated with water vapor and that water vapor behaves in accordance with the Charles law.

TABLE 1. RESIDUAL LUNG VOLUME DETERMINATIONS ON SIX TRAINED SUBJECTS EMPLOYING DILUTION OF RESIDUAL NITROGEN WITH OXYGEN OR ITS REPLACEMENT BY HELIUM, AND MEASUREMENT OF EXPANSION OF GAS IN RESIDUAL LUNG VOLUME OCCURRING IN RAPID REDUCTION OF BAROMETRIC PRESSURE FROM FOUR TO ONE ATMOSPHERES

METHOD	SUBJECTS					
	Wil	Squ	Dun	Crk	Smt	Wes
Helium substitution method	1350	1489	1584	1823	1658	
	1369	1337	1488	1701	1801	
	1160	1126				
	1133	1304				
	1158	1343				
Mean	1234	1319	1536	1762	1729	
N ₂ dilution with oxygen	1502	1345				
	1507	1339			1675	1584
	1360				1647	1717
	1437				1958	
	1586				2002	
	1401					
	1301					
Mean	1442	1342			1820	1650
Volume expansion	1418	1028	1668	1992	1760	1566
	1620	1327	1466	1576	1685	1578
	1297	1169	1558	1431	1688	1466
	1344	1384	1356		1631	1388
	1336	1126	1323			
	1113		1376			
	1248		1230			
	1162					
Mean	1317	1206	1425	1666	1691	1499

Means of values: Helium substitution method, 1427; volume expansion, 1429; nitrogen dilution, 1491.

The technic could be improved *a*) by making determinations of residual air volume following measurements of vital capacity that were in close agreement and *b*) by analyzing samples of alveolar air for inert gas, oxygen and CO₂ content.

DISCUSSION

The results of determinations on six trained subjects are recorded in table 1. It was found that measurements in very close agreement occurred most frequently

in immediately successive experiments. This was true also for vital capacity measurements.

The mean residual lung volume of the six subjects (six determinations by all methods) was 1443 cc., values in individual tests ranging from 1113 cc. to 2002 cc. By the helium substitution method the mean was 1427 cc., with a range between 1133 cc. to 1823 cc. By the volume expansion method the mean was 1429 cc. with a range of 1113 to 1992 cc.

Those subjects having high values for residual air were almost invariably long-chested, asthenic individuals. The low values obtained were generally in short-chested, muscular individuals. No constant relationship was found between values of residual lung volume and vital capacity.

It is important that the subjects be trained. In a large number of preliminary tests on subjects selected at random and not given practice runs, there was considerable variation in measurements made on any given individual.

SUMMARY

Findings are presented on the determination of residual lung volume by two new methods: *a*) substitution of helium for the nitrogen of lung air with subsequent washing out and measurement of helium contained in the residual lung volume, and *b*) measurement of gas volume resulting from physical expansion of residual lung volume occurring during rapid reduction of barometric pressure from four to one atmospheres. While, in general, greater accuracy may be attained by employing the first method, the second is conducive to speed when large numbers of determinations are to be made due to its simplicity and the possibility of making multiple determinations at one time.

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EFFECT OF BILE DIVERSION ON FECAL FAT AND NITROGEN EXCRETION¹

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ALTHOUGH there is no doubt that steatorrhea occurs when bile is excluded from the intestine, the quantitative relationship between dietary fat and fecal fat excretion during bile exclusion has not been established. Previous studies on one dog with biliary obstruction (1), two bile fistula dogs (2), three infants with bile duct atresia (3), two patients with surgical bile fistulas (4), and seven patients with biliary obstruction (5) suggest that when bile is excluded, fecal fat excretion nearly equals dietary fat when the fat intake is low, while fecal fat is considerably less than fat intake when the latter is high. The purpose of this study was to determine quantitatively the effect of bile diversion on fecal fat excretion in dogs as a step in clarifying the rôle of bile in fat absorption, and as a basis for evaluating bile replacement measures.

METHODS

Six normal dogs were given diets consisting of 'Pard' (22 per cent protein, 6 per cent fat, 5 per cent crude fiber) and lard in various proportions. Each regime was fed for seven days. All feces were pooled the last five days and analyzed for total fat and free fatty acids (6) and for nitrogen (Kjeldahl).

The same animals plus three in addition were then cholecystonephrostomized (7). After recovery the preoperative regimes were repeated and several additional regimes were tested, including a fat-free diet containing 200 grams casein and 200 grams sucrose per day. Fecal urobilinogen determinations were consistently negative on all operated dogs, and none developed jaundice.

The order of the various tests was altered among the dogs. The original plan to subject each dog to all regimes was not completed because four of the dogs developed duodenal ulcers. Statistical methods used in evaluating results are from Snedecor (8).

RESULTS

Total fecal fat. The six normal dogs excreted an average of 3.51 grams \pm 0.73 grams (st. dev.) fecal fat per day. The quantity excreted was found to be independent of the amount of fat in the diet, which varied from 15 to 47.5 grams per day. In nine dogs after cholecystonephrostomy, however, fecal fat excretion increased from 2.7 grams per day to 33.3 grams per day as daily dietary fat was increased from 0 to 51 grams. Individual five-day tests on normal and bile fistula dogs are plotted in figure 1. Dietary fat and crude fiber intakes are indicated.

Received for publication April 5, 1948.

¹ Aided in part by a grant from G. D. Searle Company, Chicago, Illinois

Since it appeared that in bile fistula dogs, diets high in crude fiber may have caused greater fecal fat excretion, independent of the effect of dietary fat, analysis by the method of multiple regression was done. The multiple regression coefficient for fecal fat on dietary fiber was not significant; hence the regression equation for fecal fat on dietary fat does not contain a third variable for crude fiber (table 1 and fig. 1).

TABLE 1. REGRESSION AND CORRELATION DATA

TYPE OF DOG	NO. OF DETERMINATIONS	REGRESSION EQUATION, $\hat{Y} = a + bX$				CORRELATION COEFFICIENT
		\hat{Y}	X	a	b \pm st. error	
Normal	17	Fecal fat	dietary fat	—	—	-0.029
Bile fistula	48	Fecal fat	dietary fat	3.70	0.583 ¹ \pm 0.079	0.909
Normal	20	% fecal FFA	dietary fat	—	—	0.080
Bile fistula	48	% fecal FFA	dietary fat	77.2	0.261 ¹ \pm 0.020	0.523
Normal and bile fistula	47	Fecal N ₂	dietary fiber	0.39	0.106 ¹ \pm 0.011	0.816
Normal	20	Fecal N ₂	dietary protein	—	—	-0.613
Bile fistula	27	Fecal N ₂	dietary protein	—	—	-0.245

¹ Significant regression coefficient at 1% level.

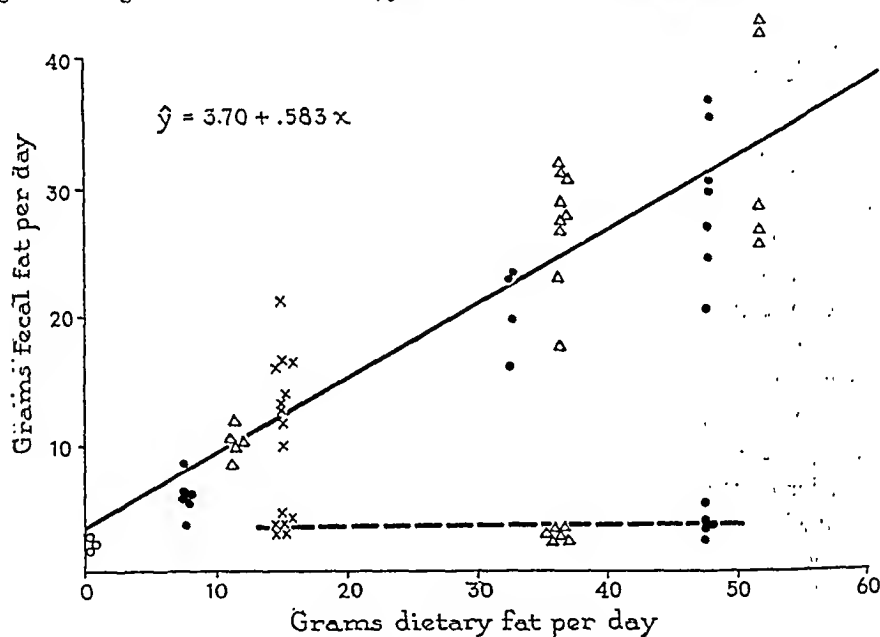


Fig. 1. RELATIONSHIP BETWEEN FECAL FAT EXCRETION AND DIETARY FAT. — = 9 bile fistula dogs; --- = 6 normal dogs; O = no crude fiber in daily diet; ● = 6 grams crude fiber in daily diet; Δ = 9 grams crude fiber in daily diet; X = 12 grams crude fiber in daily diet.

The regression equation, $Y = 3.70 + .583 \pm .079, X$ based on 48 determinations, means that in 9 bile fistula dogs, fecal fat excretion was 58 per cent \pm 8 per cent of the fat intake plus 3.7 grams. Mean daily fecal fat excretion of 3 bile fistula dogs on a fat free diet was 2.7 grams, in fair agreement with the intercept value of 3.7 grams (fig. 1).

Fecal free fatty acids. In six normal dogs fatty acid excretion did not change significantly when dietary fat was increased from 0 to 47.5 grams per day.

In nine bile fistula dogs fatty acid excretion increased more rapidly than did total fat when dietary fat was increased from 0 to 51 grams per day, so that the percentage of free fatty acids approached 100. Individual five-day tests on bile fistula dogs and means of tests on normal dogs are plotted in figure 2. Regression and correlation data appear in table 1.

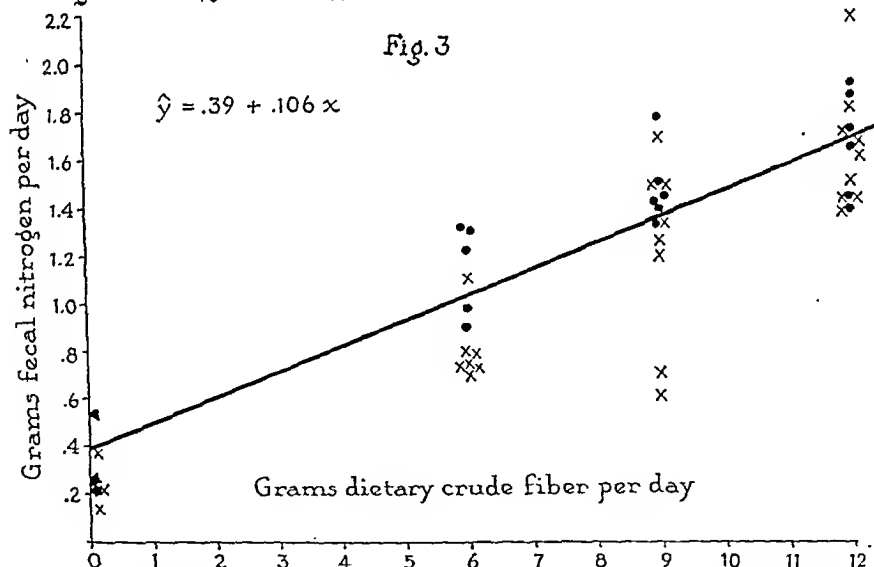
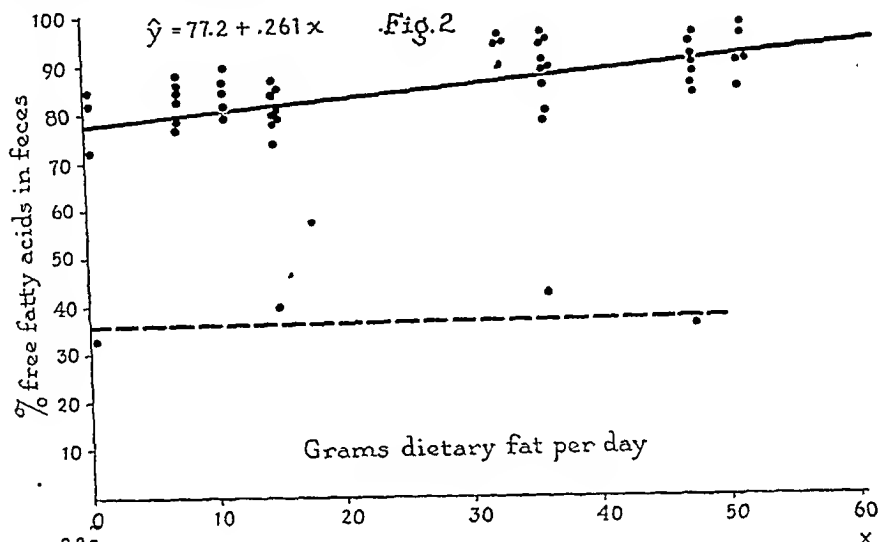


Fig. 2. RELATIONSHIP BETWEEN FREE FATTY ACID EXCRETION AND DIETARY FAT. — = 9 bile fistula dogs; --- = means of 6 normal dogs.

Fig. 3. RELATIONSHIP BETWEEN FECAL NITROGEN EXCRETION AND DIETARY CRUDE FIBER. ● = 6 normal dogs; X = bile fistula dogs.

Since fatty acids were determined in milliequivalents, then expressed as having an average molecular weight of 284 (11), the results may mean that as dietary and fecal fat increased, in the absence of bile, relatively more short-chain fatty acids are present in the feces. In the animals studied, loss of bile can be identified by the percentage of split fat in the feces, even at 0 fat intake. It is evident that bile does not furnish an essential activator of pancreatic lipase.

Fecal nitrogen. In both normal and bile fistula dogs the daily fecal nitrogen excretion varied with the different diets employed, conceivably due to changes in dietary fat, protein, and/or fiber (9), or to cholecystonephrostomy.

Nitrogen excretions were not significantly different, however, on regimes differing only in dietary fat intake (analysis of variance). Fecal nitrogen excretion was found to be correlated negatively with dietary protein and positively with dietary crude fiber (table 1). Multiple regression analysis revealed that only crude fiber was a significant factor.

In figure 3 fecal nitrogen excretion is plotted against dietary crude fiber for both normal and bile fistula dogs. The effect of crude fiber is apparent. In addition, the data demonstrate that bile diversion does not alter fecal nitrogen excretion. The regression coefficients for fecal nitrogen excretion on dietary fiber did not differ significantly between normal and bile fistula dogs (co-variance analysis). Therefore, one regression equation calculated from the two sets of data is shown in figure 3.

DISCUSSION

Previous reports have shown that normal persons excrete a constant amount of fat independent of dietary fat intake (10), and that patients with bile excluded from the bowel excrete excessive total fat and percentage of free fatty acids, but normal amounts of fecal nitrogen (5, 11). The present results agree with these reports. Coffey *et al.* (1) concluded that fecal nitrogen excretion in a dog with biliary obstruction was increased, since daily fecal nitrogen was 182 per cent of dietary nitrogen. It can be calculated from the protein intake during the test that daily fecal nitrogen excretion was only about 1.7 grams per day, which cannot be considered abnormal.

The present data on fecal fat excretion indicate that when bile fistula dogs are fed fat, fecal fat excretion is numerically equal to the amount of fat excreted on a fat-free diet (3.7 grams) plus a constant proportion (0.58) of the dietary fat. The simplest interpretation of this relationship between dietary and fecal fat is that the latter consists of a constant amount of endogenous fat plus a constant unabsorbed proportion of dietary fat. In the absence of adequate studies using labeled fat molecules this interpretation remains unproved. However, it has been reported that fecal fat excretion occurs in normal humans (12) and dogs (13) as well as in bile fistula dogs (2) on fat-free diets. Furthermore, Shapiro *et al.* (4) have shown by feeding deuterium fatty acids to patients with bile fistulas that fecal fat consists only in part of dietary fat.

It is evident from figure 1 that only when fat intake is about 9 grams per day is fecal fat excretion numerically equal to fat intake in bile fistula dogs. At lower levels of dietary fat, fecal fat excretion exceeds fat intake, and at higher levels of dietary fat the ratio of fecal fat to fat intake progressively decreases. This demonstrates that the ratio of excreted fecal fat to dietary fat is not an indication of the completeness of bile diversion, and explains why in previous reports, fecal fat, expressed as percentage of dietary fat, decreases when dietary fat is increased and bile is diverted from the intestine.

That considerable absorption of dietary fat occurs in the complete absence of bile has escaped previous general recognition and is unexplained by present theories concerning the mechanism of fat absorption.

The relationship between dietary fat intake and fecal fat excretion may be used to evaluate bile-replacement measures. The regression coefficient then becomes the ratio of fecal fat to dietary fat after proper correction is made for fecal fat excretion on a fat-free diet. Thus, in a recent study (14) bile fistula dogs were fed a diet containing 36 grams fat per day. Using the formula derived from the regression equation, $\frac{\text{Grams fecal fat} - 3.7}{36} = \text{corrected ratio of grams fecal fat to grams dietary fat}$,

the proportion of dietary fat which escaped absorption was 0.66 when no bile was given. When whole bile was fed, the corrected ratio was reduced to 0.26. This may be considered a 60 per cent improvement in fat absorption, attributable to the feeding of whole bile.

In future studies of bile replacement measures, it will not be necessary or advisable to use only a single level of fat intake, since therapy may be more effective at one level of dietary fat than at another.

SUMMARY

Fecal fat and nitrogen excretions were determined in dogs before and after cholecystonephrostomy when the mixed diet contained from 0 to 51 grams fat per day and 0 to 12 grams crude fiber per day. Tests were of five days' duration.

In 6 normal dogs daily total fat and the percentage of free fatty acids excreted in the feces remained constant and independent of fat or fiber intake. In 9 bile fistula dogs, daily fecal fat excretion increased in linear fashion as dietary fat was increased. The mathematical relationship between fat intake and fecal fat excretion was interpreted as indicating that fecal fat consists of a constant amount of fat equal to that excreted on a fat-free diet plus 58 per cent of the dietary fat.

The ratio of free fatty acid to total fecal fat was significantly higher in bile fistula dogs than in normal dogs at dietary fat levels of 0 to 51 grams per day. Daily fecal nitrogen excretion was related to the amount of crude fiber in the diet in normal and in bile fistula dogs and was independent of dietary fat, protein or of bile diversion.

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CHANGES IN THE BLOOD LEUCOCYTE LEVEL OF ADRENAL-ECTOMIZED AND NORMAL RATS FOLLOWING ADMINISTRATION OF TYPHOID VACCINE

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THE protective action of adrenal extracts against the toxic effect of typhoid vaccine in adrenalectomized rats has been reported (1). The question then arose as to the effects of such treatment on circulating white blood cells, especially lymphocytes. Dougherty and White (2) have shown that injection of adrenotropic hormone into normal mice and rats resulted in decrease in the total number of leucocytes. Relative lymphopenia and increase in polymorphonuclear leucocytes also occurred. Similarly treated adrenalectomized mice showed no lymphopenia or leucopenia but after administration of adrenal cortical extract in either normal or adrenalectomized mice, the number of lymphocytes and leucocytes fell and the polymorphonuclear leucocytes rose. The lymphopenia was interpreted as a direct action of adrenal hormone, while the increase in polymorphonuclear leucocytes was thought to be a 'nonspecific' response. They suggested that the changes in lymphocytes observed during infection might be mediated by variations in activity of the adrenal glands induced by toxic stimulation.

We wished to determine the effect of standard doses of typhoid vaccine on the white blood cells in adrenalectomized animals treated with sodium chloride, adrenal extract (aqueous alcoholic solution) or compound A acetate. The observed changes would then be ascribable directly either to absence of adrenal cortical hormone or to administered hormone.

METHODS

Fifty-six adrenalectomized rats were prepared for study as previously described (1). Male rats of the Sprague-Dawley strain weighing between 80 and 120 grams were used in all experiments. Following adrenalectomy, they were given 0.9 per cent sodium chloride solution to drink and a period of at least five days was allowed to elapse before the studies were made. Dehydrocorticosterone acetate in oil was injected daily subcutaneously. Adrenal extract was given twice daily. Blood counts were taken on 13 rats on the third day of hormone treatment, before and two hours after hormone injection. In the other hormone-treated animals 1.33 M.L.D. of typhoid vaccine was administered intraperitoneally immediately after the third morning injection of hormone. The M.L.D. was determined on adrenalectomized rats (1). Blood was taken from the tail for white blood cell, differential count and hemoglobin estimation, immediately before injection of hormone, or hormone and vaccine, and again one and two hours later. Smears for differential leucocyte counts were

also taken 45 minutes after vaccine administration. At least 200 cells were counted for each differential leucocyte count. Some rats received no replacement therapy except 0.9 per cent saline to drink. Blood counts were taken at 8:30 and 10:30 A.M. Twenty-three of these were injected with 1.33 M.L.D. typhoid vaccine immediately after the first morning bleeding. Five received an injection of one ml. saline. Some of the vaccine-injected rats were moribund at the time the 120-minute sample was taken, hence this was selected as the time for the final bleeding. By warming the animals slightly, no difficulty in obtaining the blood was encountered. We believe strain on the animals was minimal.

Thirteen normal male rats were also studied at similar time intervals following administration of typhoid vaccine. Eight received the dose found lethal to normal rats of like age and size. Five received one third more than the dose found lethal to the adrenalectomized rats, the amount used as the standard test dose in determining the toxic protection power of adrenal extracts in adrenalectomized rats. To control the experiment still further, groups of adrenalectomized, hormone-treated animals and groups receiving only sodium chloride were studied simultaneously and the data on the corresponding groups compared.

RESULTS

The average leucocyte count of adrenalectomized rats receiving either compound A acetate or adrenal extract was slightly lower than that of those receiving saline (26, 29 and 30 thousand per cu. mm. for compound A acetate, adrenal extract and saline, respectively). The hormone-treated animals had a somewhat higher percentage of polymorphonuclear neutrophils (23 per cent) than those treated only with sodium chloride (17 per cent). Sixty minutes after receiving typhoid vaccine, both the hormone and saline-treated groups usually showed some decrease in total leucocyte count with relative lymphocytosis. In some instances, the greatest percentage of lymphocytes was found 45 minutes following administration of toxin, whereas in others, an even greater percentage was observed at 60 minutes (fig. 1). This difference is possibly due to variations in the rate of absorption of the toxin.

Little change in the blood leucocyte or lymphocyte level of adrenalectomized rats was observed two hours after injection of adrenal extract, compound A acetate or saline (fig. 2). In contrast with the minimal changes observed in these groups, the animals showed large shifts two hours after receiving typhoid vaccine and hormone, or typhoid vaccine and saline. The total leucocyte count of the hormone-toxin-treated animals showed only slight changes. The large decrease in circulating lymphocytes in the hormone-treated animals was accompanied by an increase in polymorphonuclear neutrophils (7.9 thousand per cu. mm. in the adrenal extract-treated animals and 8.3 thousand per cu. mm. in the compound A acetate-treated group). On the other hand, the saline-treated animals which received typhoid vaccine showed an average decrease in leucocyte count of 5.3 thousand per cu. mm. and an average increase in the polymorphonuclear neutrophils of only 4.6 thousand per cu. mm.

All of the animals receiving hormone injections were alive 24 hours after injection of typhoid vaccine, while all those treated only with saline died.

No significant change in level of hemoglobin was observed following typhoid vaccine injection in the animals receiving adrenal extract or compound A acetate.

It ranged from 12 to 14 grams per 100 ml. of blood. In contrast with the hormone groups, the saline-treated animals showed definite increase in hemoglobin concentration two hours following typhoid vaccine. From an average level of 13.5 grams before toxin, it rose to an average of 15.5 grams per 100 ml. of blood. It is interesting that despite hemoconcentration, the blood leucocyte count decreased in the saline-treated group, whereas it remained unchanged in the hormone-treated groups which showed no hemoconcentration.

The leucocyte counts on the adrenalectomized rats were higher than might be expected in animals free of infection. However, all of them appeared healthy and showed weight gain. Furthermore, the control and hormone-treated animals were

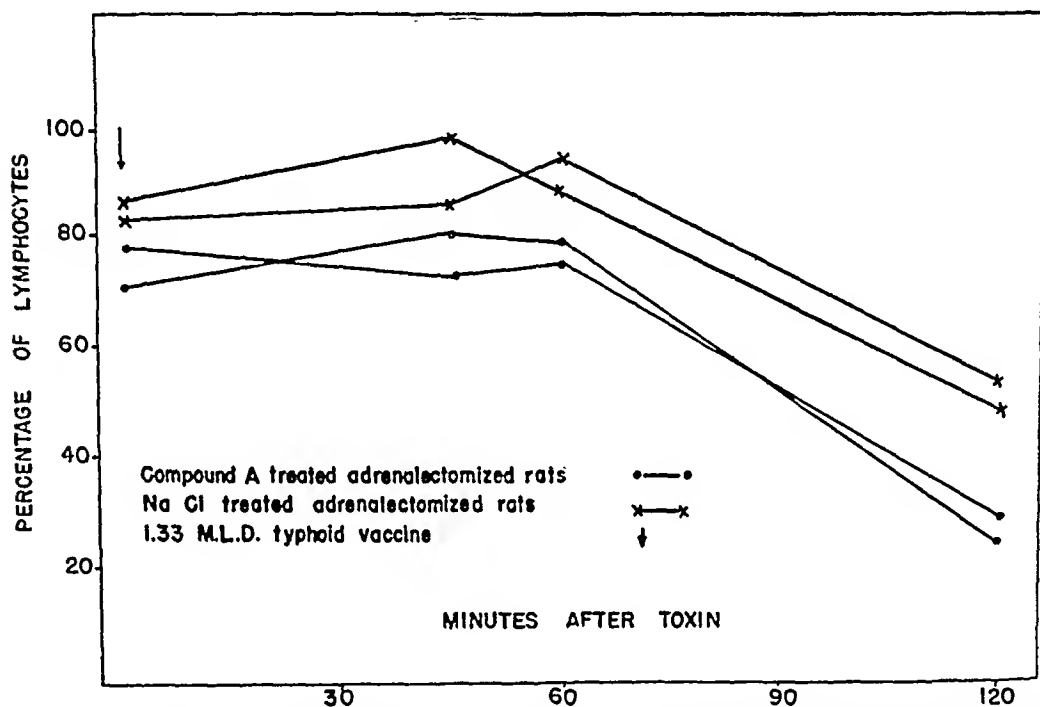


FIG. 1. CHANGE IN THE PERCENTAGE OF LYMPHOCYTES in the blood of adrenalectomized extract-treated and adrenalectomized saline-treated rats 45 minutes, 60 minutes and 120 minutes after the intraperitoneal injection of 1.33 M.L.D. typhoid vaccine.

studied simultaneously. Each animal served as its own control, as the blood count obtained immediately before injection of hormone or saline and typhoid vaccine, or of hormone and saline alone, was compared with the count obtained two hours later.

Normal rats which received a lethal dose of typhoid vaccine showed a somewhat greater decrease in the leucocyte count two hours after administration of vaccine than normal animals which received a sublethal dose (1.33 M.L.D., the M.L.D. being determined on adrenalectomized rats), figure 3. The difference between the two groups was not as great as between the hormone and nonhormone treated adrenalectomized animals. It is possible that greater differences would have been found had counts been taken nearer the time of death in the lethal dose group. In normal animals death usually occurred 10 to 24 hours following one M.L.D. of toxin (M.L.D. as determined on normal animals) while in adrenalectomized rats death usually took

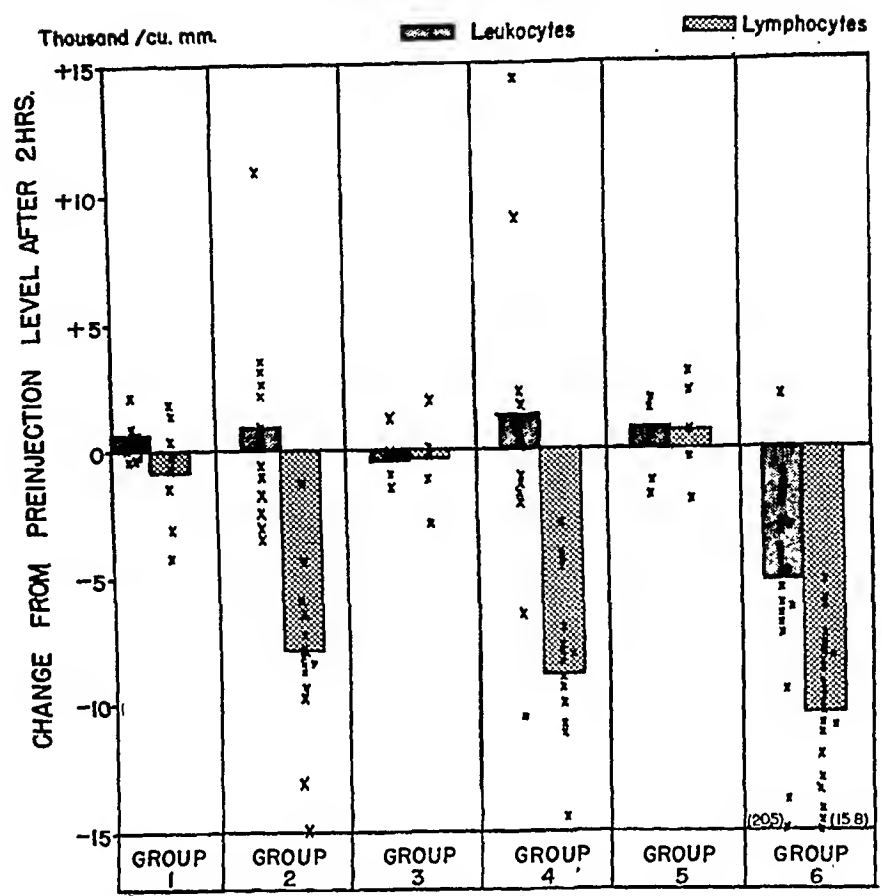


FIG. 2. CHANGE FROM PREINJECTION LEVEL in the blood leucocyte and lymphocyte count of adrenalectomized rats two hours after injection of 1.0 ml. adrenal extract, *group 1*; 1.0 ml. adrenal extract and 1.33 M.L.D. typhoid vaccine (intraperitoneal) *group 2*; 0.4 mgm. compound A acetate, *group 3*; 0.4 mgm. compound A acetate and 1.33 M.L.D. typhoid vaccine (intraperitoneal), *group 4*; 1.0 ml. 0.9 per cent sodium chloride solution, *group 5*, and 1.0 ml. 0.9 per cent sodium chloride solution and 1.33 M.L.D. typhoid vaccine (intraperitoneal), *group 6*.

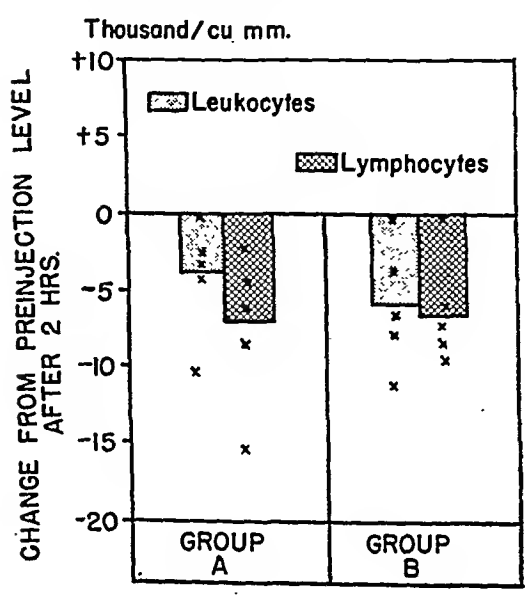


FIG. 3. CHANGE FROM PREINJECTION LEVEL in the blood leucocyte and lymphocyte count of normal rats two hours after the intraperitoneal injection of 1.33 M.L.D. (as determined on adrenalectomized rats) typhoid vaccine, *group A*, and two hours after the intraperitoneal injection of 1.0 M.L.D. (as determined on normal rats) typhoid vaccine, *group B*.

place 3 to 6 hours after 1.33 or 1.0 M.L.D. typhoid vaccine (M.L.D. as determined on adrenalectomized rats).

Shifts in the eosinophile level following typhoid vaccine injection in both normal and adrenalectomized animals were small, but tended to show a slight increase in percentage and absolute number.

Blood counts made on a series of 16 normal people before and four hours after receiving immunizing doses of typhoid vaccine showed a marked leucocytosis with a relative and absolute lymphopenia. The changes were similar to those observed in adrenalectomized-adrenal hormone-treated rats and in normal rats after injection of typhoid vaccine. Whether the leucocyte pattern in human beings with decreased adrenal cortical activity would show shifts following typhoid vaccine similar to that of adrenalectomized-typhoid vaccine-treated rats is still to be investigated.

DISCUSSION

The very rapid changes in the white blood cells which occur following typhoid vaccine administration in saline-treated adrenalectomized animals provides evidence that, at least for a short time, certain important shifts can occur even in the absence of adrenal cortical hormone. The rapid decrease in circulating lymphocytes can occur without mediation of administered adrenal hormones. On the other hand, the large increase in circulating neutrophils following toxin appears to be in part dependent on their presence.

SUMMARY

Adrenalectomized rats treated for two days with either adrenal extract or compound A acetate showed no significant change in blood lymphocytes or leucocytes two hours after injection of these hormones. Nor did saline-maintained rats show changes after saline injection.

Intraperitoneal injection of typhoid vaccine markedly lowered the lymphocytes two hours after injection, whether or not the rats had been previously treated with adrenal extract, compound A acetate or saline. In contrast, the polymorphonuclear neutrophils increased. This increase was significantly greater in the hormone-treated animals.

Normal rats and human beings injected with typhoid vaccine showed a decrease in lymphocytes and increase in polymorphonuclear neutrophils, similar to that observed in adrenalectomized-adrenal hormone-treated rats following injection of typhoid vaccine.

It is concluded that the lymphopenia elicited by typhoid vaccine is not dependent on adrenal cortical hormones, but the concurrent polymorphonuclear leucocytosis is augmented by their presence.

We thank Dr. Randolph Major of Merck and Company for the dehydrocorticosterone acetate, Dr. M. Kuizenga of Upjohn Company for the adrenal extracts and Mrs. Irene Smith Sparks for valuable help on the blood counts.

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CHANGES IN ARTERIAL INFLOW IN THE DOG'S LEG FOLLOWING VENOUS OCCLUSION: EVALUATION OF RESULTS OBTAINED WITH DIFFERENT TYPES OF FLOW RECORDERS¹

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CLINICAL experience has generally supported the belief that a limb deprived of its main arterial blood supply is benefited by surgical ligation of its main vein. The circulation of blood through the limb would appear to be enhanced by the procedure, inasmuch as the incidence of gangrene is reported to be less in limbs subjected to ipsilateral venous ligation operations.

Various theories have been advanced to explain these results. Anatomical studies of injected specimens suggest that the finer blood vessels increase in size without appreciable increase in their number (1). The more recent views on the subject are contained in the papers of Linton, Morrison, Olfeder and Libby (2), and Friedland, Hunt and Wilkins (3). The former group has reported that partial obstruction of the venous outflow from the leg of the dog caused a prompt and considerable increase in blood flow through the femoral or iliac artery when measured with a thermostromuhr. The increase in arterial blood flow was reported to occur during the period of congestion, rather than after the release of venous compression, and hence they have advocated prolonging the period of venous stasis when intermittent venous occlusion is used. The latter group (3), using a plethysmograph on the legs of normal patients, has indicated that a reduced arterial inflow occurred during venous compression from the application of a tourniquet.

Since present views differ with respect to the immediate blood flow benefits of such a procedure, animal experiments were performed using several different instruments to determine the changes in arterial inflow to a limb which might occur with partial venous occlusion. It was hoped that such a study might also help to explain why different previous methods have yielded results which are diametrically opposed. For this reason, the blood flows were measured by the rotameter, orifice meter and thermostromuhr.

METHODS

The experiments were performed on 30 mongrel dogs ranging from 10 to 15 kilograms in weight. After preoperative administration of morphine sulfate, and either sodium pentobarbital or sodium amytal, a leg artery and vein (iliac or femoral) were exposed and isolated.

In some experiments, after injection of pontamine fast pink (150 mgm/kilo) and heparin (1 mgm/kilo), the rate of arterial inflow was recorded by an optically recording rotameter (4, 5) or orifice meter (6, 7) inserted between the cut ends of the artery. In other experiments, the Baldes-Herrick type of thermostromuhr (8) was used alone

Received for publication March 25, 1948.

¹ Expenses of this investigation were defrayed by a grant from the Commonwealth Foundation.

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to measure arterial inflow, or was applied to the same artery in series with the rotameter or orifice meter. The thermostromuhr was usually calibrated *in situ* if it had been used to measure flow simultaneously with the rotameter. To do this, the outflow from the rotameter (which was downstream from the thermostromuhr) was shunted by means of a rubber tube to the femoral vein of the opposite leg and the rate of blood flow adjusted manually by a screw clamp during the calibration. Occasionally, the thermostromuhr calibration was performed on the same vessel removed to an artificial circulation system.

In those experiments in which the thermostromuhr and rotameter were used to measure flow simultaneously in the same artery, the rotameter flow values were determined by reading the height of the rotameter float at regular intervals (9), the time being indicated on the camera. The nonrecording type of rotameter was used in these instances, since it has a minimal damping effect on the phasic oscillations of the flow pulse. As desired, mean or phasic arterial pressures were optically recorded (10, 11) from a side connection on the rotameter or orifice meter cannula, while the venous pressure was similarly recorded from a T-tube inserted in the femoral vein.

Elevation of venous pressure was induced by clamping either the femoral or iliac vein of the limb or by placing a rubber tourniquet around the thigh under the femoral artery in which the flow was being measured. Elevation of venous pressure was maintained continuously for one to 15 minutes.

The arterial blood flow was measured either with the natural limb temperature prevailing or with the limb temperature and/or its arterial flow artificially elevated by the use of heat lamps, hot packs or by recent sciatic nerve section or block.

The flow changes as shown by the rotameter, orifice meter and thermostromuhr will be considered separately.

RESULTS

1. *Rotameter.* Figure 1 illustrates the typical changes in the mean rate of flow through the femoral artery as recorded by an optically recording rotameter during and after temporary occlusion of the ipsilateral femoral vein. Flow in the femoral artery at first decreases considerably and then partially returns toward the control flow level. Immediately following release of the venous clamp, the arterial flow rises temporarily to exceed the control value. In no experiment has the partial restoration of flow which occurs late during the elevation of venous pressure ever approximated the control flow. Usually, this trend was less than that illustrated in figure 1. The increase in blood flow following release of the venous clamp has never been found to compensate quantitatively for the flow deficit incurred during the period of venous pressure elevation. The application and release of a leg tourniquet caused the same directional changes in flow as with a single vein occlusion.

In other experiments using the rotameter, similar qualitative flow responses occurred when flow was measured in the iliac artery during temporary occlusion of the ipsilateral iliac or the femoral vein.

Under conditions in which the control arterial blood flow was greatly increased either by warming the limb to a temperature above normal or by section or block of the sciatic and femoral nerves, subsequent venous occlusion or application of a tourniquet caused the same directional flow changes as before, but the flow depression

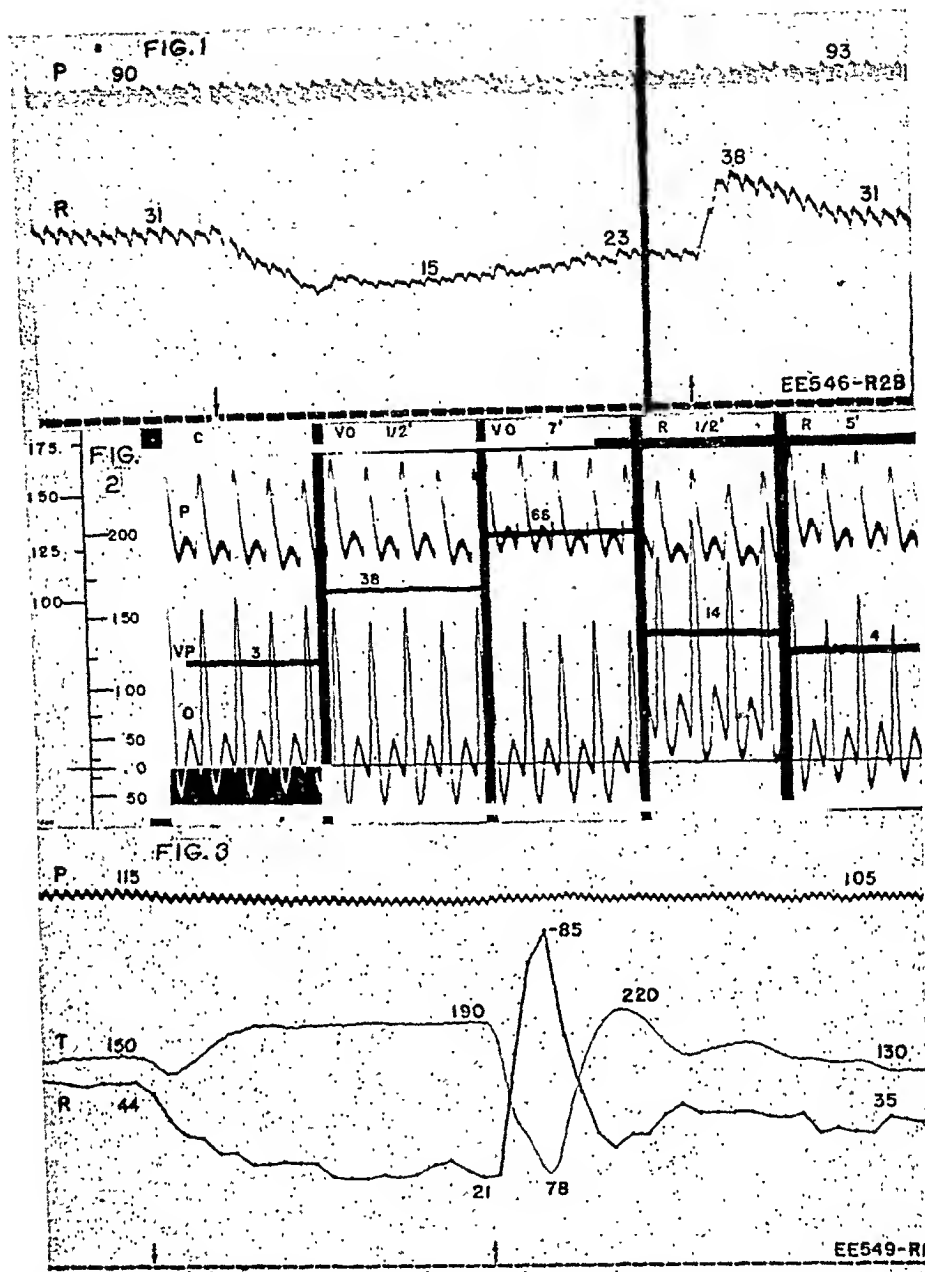


Fig. 1. PHOTOGRAPH OF ORIGINAL RECORD SHOWING THE CHANGES IN MEAN FLOW in the femoral artery (as recorded by the optically recording rotameter) during occlusion (\downarrow) and release (\uparrow) of the ipsilateral femoral vein. Vertical intercept, one-minute period of venous occlusion deleted. Upper curve (P), mean femoral artery blood pressure; lower curve (R), mean femoral artery flow as recorded with the rotameter; time interval, 5 seconds.

Fig. 2. REPRODUCTION OF SEGMENTS OF A SERIES OF ORIGINAL RECORDS showing the effect of femoral vein occlusion and release on phasic flow in the ipsilateral femoral artery. Ordinate scales, pressure in millimeters of mercury on left, flow in cubic centimeters per minute on right. Continuous narrow horizontal line in flow pattern indicates zero flow. Letters on each record segment are: top curve (P), femoral artery pressure; middle curve (VP), mean femoral vein pressure; lowest curve (O), velocity curve in femoral artery recorded by orifice meter; C, control record; VO, vein occlusion; R, vein release. Numbers on each record segment are: at top, time elapsed following venous occlusion and release of vein; middle, venous pressure.

Fig. 3. PHOTOGRAPH OF ORIGINAL RECORD SHOWING MEAN FLOW CHANGES in the femoral artery during occlusion and release of the femoral vein. Upper curve (P), mean femoral artery pressure; middle curve (T), mean flow as recorded with the thermostromuhr; lowest curve (R), mean flow as indicated with the rotameter; time interval, 5 seconds.

during venous occlusion and the flow augmentation after venous release were generally greater (records not shown).

2. *Orifice meter.* Figure 2 illustrates the phasic flow pattern and mean blood flow changes in the femoral artery during occlusion and release of the femoral vein. Again, mean flow decreases during venous occlusion and increases following release. In the control record, a sizeable back flow component is present during the early part of diastole. This is the usual type of flow curve obtained in the femoral artery. The effect of venous occlusion is observable almost immediately in the phasic flow curves. Thirty seconds after venous clamping and with the femoral venous pressure at 38 mm. Hg, the diastolic back-flow component is increased considerably in rate and volume, while the forward flow in both systole and diastole is decreased. Maintenance of venous occlusion up to seven minutes, with elevation of venous pressure to 66 mm. Hg, does not materially increase the back-flow component or reduce the forward flow in systole and diastole. Within 30 seconds following removal of the venous occlusion, and as the venous pressure returns toward normal, the back-flow component disappears as both systolic and diastolic flows increase greatly. The heart rate remained constant at 178 to 180 per minute.

Similar flow pattern and volume flow changes occurred during venous occlusion when the sciatic nerve had previously been sectioned to increase the control flow and to decrease its back flow component.

3. *Thermostromuhr with rotameter or orifice meter.* Blood flow in the femoral artery during local venous occlusion was recorded by the thermostromuhr alone, or simultaneously by the thermostromuhr and either the rotameter or orifice meter. The flow trends were identical in all experiments. Reproduction of a typical record in which flow was indicated by the rotameter and thermostromuhr is shown in figure 3. As in figure 1, the arterial flow to the limb as measured by the rotameter diminishes during venous occlusion, rises temporarily to exceed the initial level after flow through the femoral vein is reestablished and then returns essentially to the control level. The control thermostromuhr arterial flow, however, is of considerably greater magnitude, 150 cc. versus 44 cc. per minute, and coincident with elevation and reduction in local venous pressure, the flow apparently increases and decreases, respectively. In general, the thermostromuhr flow record is a mirror image of the rotameter flow curve.

DISCUSSION

The present experiments demonstrate that, coincident with the elevation and subsequent reduction of venous pressure in an extremity, the arterial inflow decreases and then increases, respectively, when measured by the rotameter and orifice meter, while the thermostromuhr indicates that the inflow first increases and then decreases during this procedure.

As reported in previous communications (4-6, 7, 9), tests have shown that the rotameter and orifice plate meter are adequate for quantitative measurements of mean and phasic blood flow, respectively, and the absolute flow values may be accepted as being correct within a 10 per cent experimental error.

Therefore, there is no reasonable doubt that arterial blood flow to a limb of an experimental, anesthetized animal is acutely decreased during temporary venous occlusion of the ipsilateral limb vein. This agrees with the findings of Wilkins *et al.*

(3) that blood flow into the extremities of man, as measured by the plethysmograph, is usually reduced during local elevation of venous pressure by a tourniquet.

The apparent flow increase during venous occlusion, observed with the thermostromuhr, was invariably found by Linton *et al.* (2), but the decrease in flow following release was not always observed. The reason why the flow changes indicated by the thermostromuhr are diametric opposites of those obtained with other flow recorders remains to be explained. A reasonable explanation can be deduced from the drawing in figure 4, which has been assembled from actual records and data obtained in different experiments. The curves represent the changes in mean blood flow, phasic flow and back flow that occur simultaneously during elevation of venous

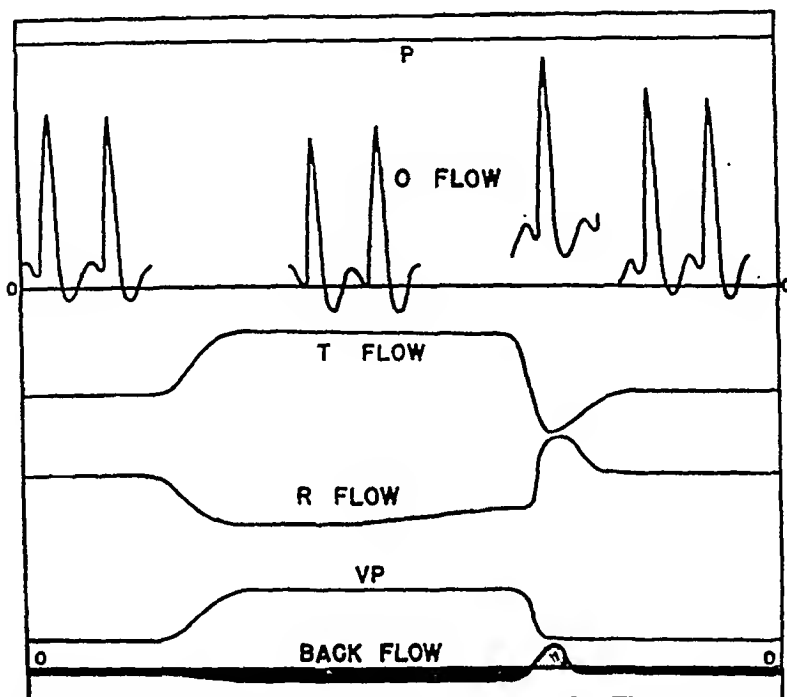


Fig. 4. SCHEMATIC DRAWING TO ILLUSTRATE THE DYNAMIC CHANGES in the vessels of extremities which are responsible for failure of the thermostromuhr to indicate correctly flow changes during local venous occlusion and release: *P*, mean blood pressure; *O* flow, phasic flow with orifice meter; *T* flow, thermostromuhr flow; *R* flow, mean arterial flow with rotameter; *VP*, mean venous pressure; *back flow*, black area; *O*, zero flow.

pressure, as recorded by the orifice meter, rotameter and thermostromuhr. It will be observed that a back flow component is normally present in the femoral artery flow pattern, is maximal during the period of greatest reduction in flow, and is largely or entirely absent during the early phase of recovery (following tourniquet release) when the flow is considerably increased. Although other mechanisms may be partially responsible, these changes in the back flow component are believed to be the main factor responsible for the failure of the thermostromuhr to indicate correctly the diminution and augmentation in flow which occur with the venous occlusion procedure. Although the thermostromuhr method is subject to a number of errors, it has been previously demonstrated that the instrument is particularly sensitive to the presence of back flow in the cycle flow pattern (12, 13). A very small back flow

of blood during each cycle alters the differential temperature at the thermocouples in such a manner that the galvanometer indicates a much larger flow of blood through the thermostromuhr than actually exists (see fig. 3). Under conditions in which the blood flow decreases and the back-flow component simultaneously increases in magnitude, the net effect upon the thermostromuhr is a decrease in the temperature gradient between the two thermocouples so that the instrument erroneously indicates an apparent increase instead of an actual decrease in blood flow. Similarly, an actual increase in blood flow sufficient to eliminate the back flow component from the flow cycle may be recorded as an apparent decrease in blood flow (fig. 3). Thus, in terms of the experiments reported here, the magnitude of the effect of small increments of back flow, indicated by the thermostromuhr as more flow, is greater than the effect of a large reduction in flow. Since, in the circumstances of these experiments, the back flow increased somewhat and the mean flow decreased greatly, this flow instrument failed to indicate properly even the directional change in flow.

SUMMARY

Experiments were performed to determine the effect of local elevation of venous pressure by tourniquet or partial vein ligation on arterial inflow to the leg of the anesthetized dog as measured by the rotameter, orifice meter and thermostromuhr. With the rotameter and orifice meter, arterial inflow was found to decrease during venous pressure elevation and to increase temporarily above the control value immediately following release of venous constriction. There is no reasonable doubt that under these conditions the immediate effect of such a procedure is a decrease in arterial inflow into a limb.

With the thermostromuhr, the inflow changes were reversed, increasing and decreasing, respectively, with venous pressure elevation and release. These results are believed to be erroneous and can be attributed largely to the inadequacy of the thermostromuhr in measuring blood flow under conditions in which back flow is present in the cyclic flow pattern.

The results of this study do not necessarily have any bearing on the therapeutic value of intermittent venous occlusion in the treatment of peripheral vascular disease.

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RENAL HYPEREMIA AFTER THE INTRAVENOUS INFUSION OF ADENYLIC ACID, ADENOSINE, OR ADENOSINE-TRIPHOSPHATE IN THE DOG

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RENAL hyperemia (diodrast or PAH clearance), occurring too rapidly to be caused by hypertrophy of renal tissue, has been produced by the injection of pyrogenic inulin and triple typhoid vaccine into man (1, 2), dog (3) and seal (4), and by the infusion of glycine (5), oral administration of sulfates of the cinchona alkaloids (6) or a high protein diet (5) in the dog. It seemed of interest to investigate whether or not a similar, more pronounced, and perhaps more consistent hyperemia could be induced in dogs by the infusion of the natural tissue metabolites, adenylic acid, adenosine and adenosinetriphosphate (ATP). This was prompted by *a*) the demonstration in chloralosed cats by Keele and Slome (7) of an occasional renal vasodilatation and hyperemia following the parenteral injection of the sodium or magnesium salt of ATP and *b*) by the suggestion that these adenine derivatives might be important in the regulation of the circulation during rest and exercise (8) and during shock produced in the 'crush syndrome' (9, 10).

EXPERIMENTAL PROCEDURE

Glomerular filtration rate and effective renal plasma flow were measured according to the technics described in a previous paper (11). Mean arterial blood pressure was obtained by means of a femoral arterial puncture and a mercury manometer.

The effects of the intravenous infusion of yeast (3-adenylic),⁵ and muscle (5-adenylic)⁶ adenylic acid, yeast adenosine,⁵ or sodium adenosinetriphosphate⁶ on filtration rate and effective renal plasma flow were studied in 10 dogs, selected from the previous control series of 75 dogs (11). After two to three control urine collection periods, a separate infusion of the adenine derivative was started in a leg vein, and, after a discard period of from 5 to 10 minutes, from 3 to 8 additional urine collections were made.

Plasma concentrations of yeast adenylic acid were determined in three experiments before, during and after the infusion of this material, by the spectrophotometric method of Kalckar (12) or Archibald (13).

The barium salt of adenosinetriphosphate was purified and converted to the sodium salt be-

Received for publication March 6, 1948.

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⁵ 3-adenylic acid and yeast adenosine from B. L. Lempke Chemical Company, New York.

⁶ 5-adenylic acid and sodium adenosinetriphosphate from Armour and Company, Chicago, Illinois.

fore infusion as follows: one gram was suspended in 5 to 10 cc. of water; 2 N nitric acid was added dropwise until all of the compound was dissolved. After chilling, 20 per cent mercuric nitrate in 0.5 N nitric acid was then added until no more precipitate was formed. The precipitate was washed twice with water and suspended in water and decomposed with hydrogen sulfide until completely

TABLE 1. FILTRATION RATE, EFFECTIVE RENAL PLASMA FLOW AND FILTRATION FRACTION, DURING, AND AFTER THE INTRAVENOUS INFUSION OF ADENYLIC ACID, ADENOSINE OR SODIUM ADENOSINETRIPHOSPHATE IN THE DOG EXPRESSED AS PER CENT OF CONTROL

DOG	BODY WEIGHT	RATE OF DELIVERY OF COMPOUND		TOTAL AMOUNT OF COMPOUND INFUSED		C _{CR}		C _{PAH}		C _{CR} /C _{PAH}		BLOOD PRESSURE		
						Dur- ing infu- sion	After infu- sion	Dur- ing infu- sion	After infu- sion	Dur- ing infu- sion	After infu- sion	Before infu- sion	Low- est dur- ing infu- sion	After infu- sion
	kgm.	mgm/ min.	mgm/ kgm/ min.	mgm.	mgm/ kgm.	per cent of control						mm. Hg		
a. Yeast Adenylic Acid (3-adenylic acid)														
53	11.2	39.4	3.53	767	68.5	100	110	111	119	78	93	141	47	104
5	17.8	26.8	1.50	938	52.7	58	95	116	145	50	66	160	95	138
22	17.5	16.0	0.92	770	44.0	75	148	124	168	62	76	130	90	120
40	22.8	74.5	3.26	895	39.2	6	101	7	168	84	60	110	40	75
16	8.8	8.1	0.92	226	25.7	71	105	114	133	63	81	—	—	—
9	12.0	19.8	1.65	416	34.7	91	118	127	140	72	85	140	80	125
16	8.8	9.7	1.10	228	25.9	99	121	155	152	65	83	130	105	130
b. Muscle Adenylic Acid (5-adenylic acid)														
40	17.0	26.8	1.58	309	18.2	54	107	108	111	50	97	128	105	—
75	15.8	12.5	0.79	232	14.7	0	183	0	200	0	80	127	72	118
		26.8	1.70	214	13.5	37	112	41	128	90	87	128	84	120
		11.1	0.70	128	8.1	60	90	82	91	119	99	122	110	118
c. Yeast Adenosine														
8	9.3	38.2	4.11	916	98.5	60	116	106	127	58	92	145	100	130
6	11.0	29.5	2.68	885	80.5	86	108	113	133	76	82	—	—	—
22	17.5	—	—	—	—	79	104	111	137	70	75	130	90	—
7	13.5	42.9	3.17	900	66.6	11	94	15	133	72	72	135	82	138
d. Sodium Adenosinetriphosphate														
40	22.8	88.6 ¹	3.89 ¹	400 ¹	17.6 ¹	2	86	2	127	100	68	120	50	—
		98.0 ¹	4.30 ¹	196 ¹	8.6 ¹	86	105	88	95	98	111	124	60	140

¹ Expressed as the dibarium salt of A.T.P.

black. The mercuric sulfide was allowed to settle rendering a clear supernatant fluid, which was filtered. The filtrate was aerated for an hour and then neutralized to a pH of 7.5 with one N sodium hydroxide. The concentration of adenosinetriphosphate in this solution equivalent to the original barium salt was calculated from the concentration of hydrolyzable phosphorus.⁷ Phosphorus was measured by the method of Fiske and Subbarow (14). For hydrolyzable phosphorus, an aliquot was heated in a boiling water bath at 100° C. for 15 minutes, after making the solution normal with respect to the hydrochloric acid.

⁷ Analyzed by Dr. George H. Hitchings at the Wellcome Research Laboratories, Tuckahoe, New York.

RESULTS AND DISCUSSION

The effects of infusion of yeast and muscle adenylic acid, yeast adenosine and sodium adenosinetriphosphate on renal function are given in table 1, which shows rates of infusion of these substances and the total amount infused, and expresses filtration rate and renal plasma flow during and for three urine collection periods after such infusions as per cent of control or the pre-infusion average for each separate observation. The relationship between glomerular filtration rate and renal plasma flow during and after infusion is expressed graphically in figure 1 as percentage of

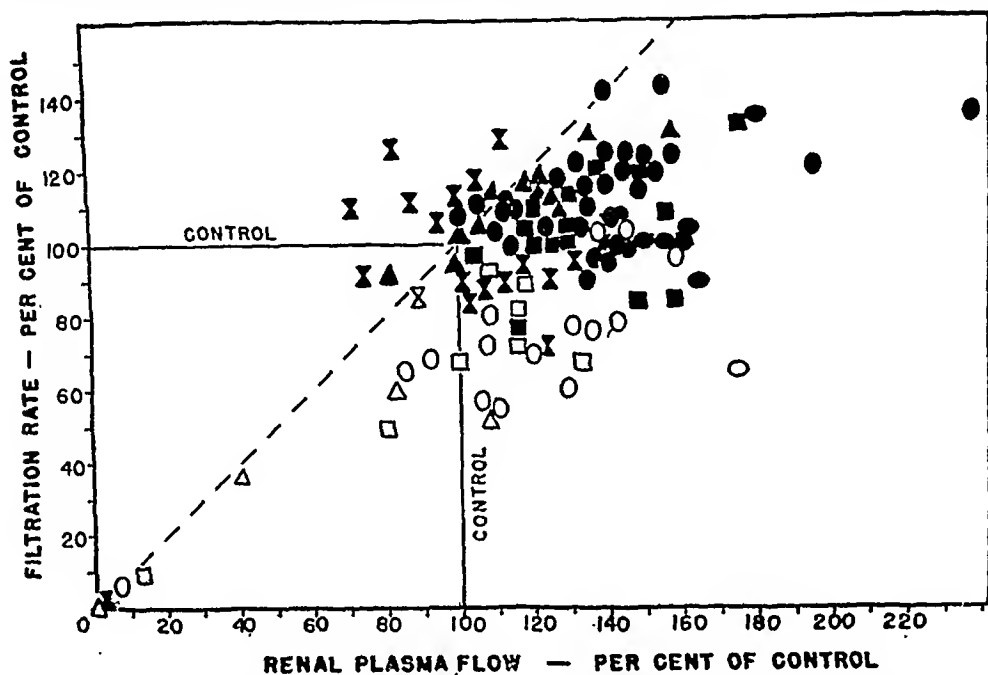


Fig. 1. RELATION OF FILTRATION RATE TO RENAL PLASMA FLOW during and after the intravenous infusion of yeast adenylic acid (*circles*), muscle adenylic acid (*triangles*), yeast adenosine (*squares*), and sodium adenosinetriphosphate (*hour glasses*) in 16 experiments on 10 dogs. Open symbols indicate the infusion phase; closed symbols, the post-infusion phase. Movement of a point along the dashed line indicates proportional changes in both functions, i.e., no alteration in the filtration fraction. Movement of a point to the right indicates a decrease, movement to the left, an increase in the filtration fraction.

control values. The time course of these effects during and after infusion of adenylic acid in three individual experiments are given in figures 2, 3, and 4.

It is apparent that these effects on renal function fall into two phases: *a*) the infusion phase and *b*) the post-infusion phase.

a) Infusion phase. The infusion phase is characterized by a variable reduction in blood pressure and a reduction in filtration rate ranging from 10 to 100 per cent, depending upon the degree of reduction in blood pressure. In the experiments where filtration is between 65 and 100 per cent of control (fig. 1, open symbols), renal plasma flow remains constant or may increase to as much as 180 per cent of control (fig. 3). However, if filtration drops below 65 per cent of control, renal plasma flow also decreases. In fact, if filtration drops to 37 per cent of control or below, the apparent renal plasma flow drops correspondingly to or very near zero in some instances (fig.

4). As long as flow is increased or is not markedly decreased, filtration fraction (filtration/flow) decreases (fig. 1, 2, 3). However, when flow is decreased to 40 per cent of control filtration is correspondingly reduced, consequently, the filtration fraction is unaffected.

The decreased blood pressure is due to a decrease in peripheral resistance caused by general arteriolar dilatation resulting from infusion of adenine derivatives (15, 16, 17). When blood pressure dropped to or below 70 mm. Hg both filtration rate and renal plasma flow were proportionally markedly reduced, to zero in some instances

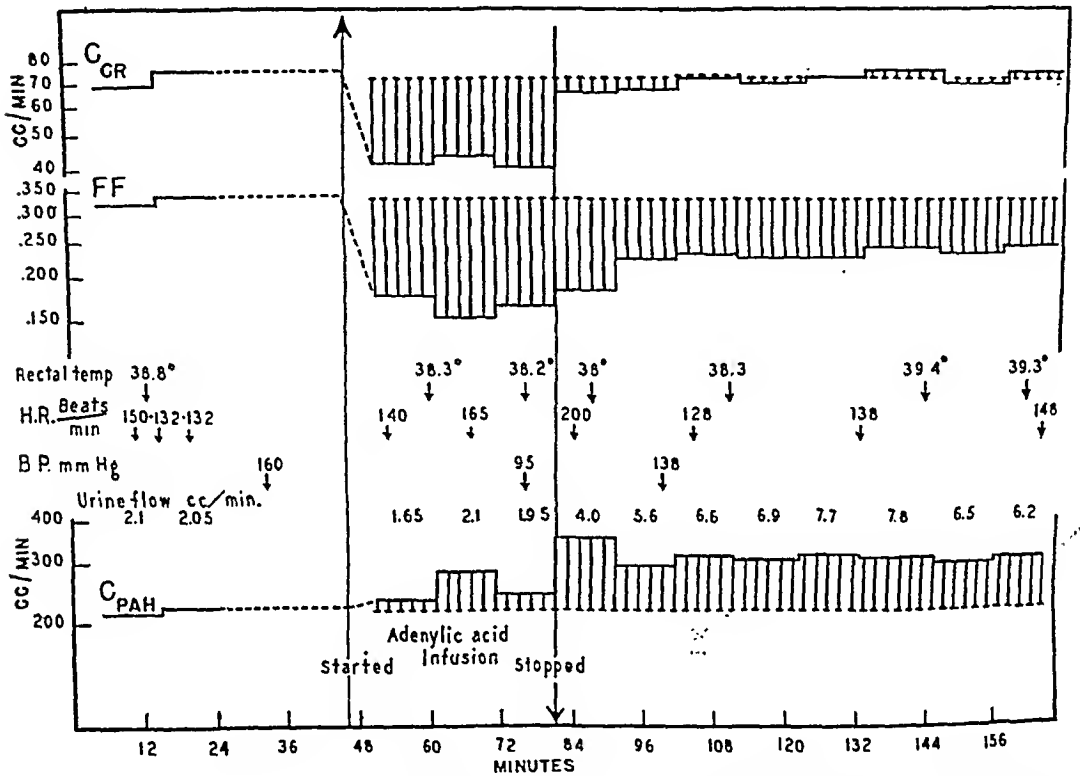


Fig. 2. EFFECTS OF THE INTRAVENOUS INFUSION OF 1.50 mgm./kgm./min. of yeast adenylic acid into a 17.8 kgm. dog for 35 minutes (total dose = 52.7 mgm./kgm.) on filtration rate, renal plasma flow, and filtration fraction, all plotted on logarithmic ordinates, and mean arterial blood pressure, urine flow, heart rate and rectal temperature.

(table 1). This can be explained by a reduction in hydrostatic pressure in the glomerulus and peritubular capillary plexus, due to a reduced blood pressure resulting from general arteriolar dilatation. As long as the blood pressure remains above 70 mm. Hg, reduced filtration can probably be attributed in part to some degree of afferent arteriolar constriction. The failure of renal plasma flow to decrease as extensively as filtration rate and the observed increase in flow coexisting with decreased filtration, resulting in a decreased filtration fraction in either case, suggest some degree of efferent arteriolar dilatation (2).

b) *Post-infusion phase.* This phase follows immediately after stopping the infusion of the adenine derivatives. It consists of an immediate return (fig. 2, 4) of blood

pressure and filtration to or near the control value, sometimes above control, and, in every instance, an increased renal plasma flow ranging from 111 to 240 per cent of control in individual urine collection periods (fig. 1, closed symbols). This hyperemia has been observed to last for as long as 90 minutes (fig. 2). Consequently, the filtration fraction remains reduced, indicating dilatation principally of the efferent glomerular arteriole and, to some degree, of the afferent arteriole (2). It is reasonable to believe that this renal arteriolar dilatation is part of the general arteriolar

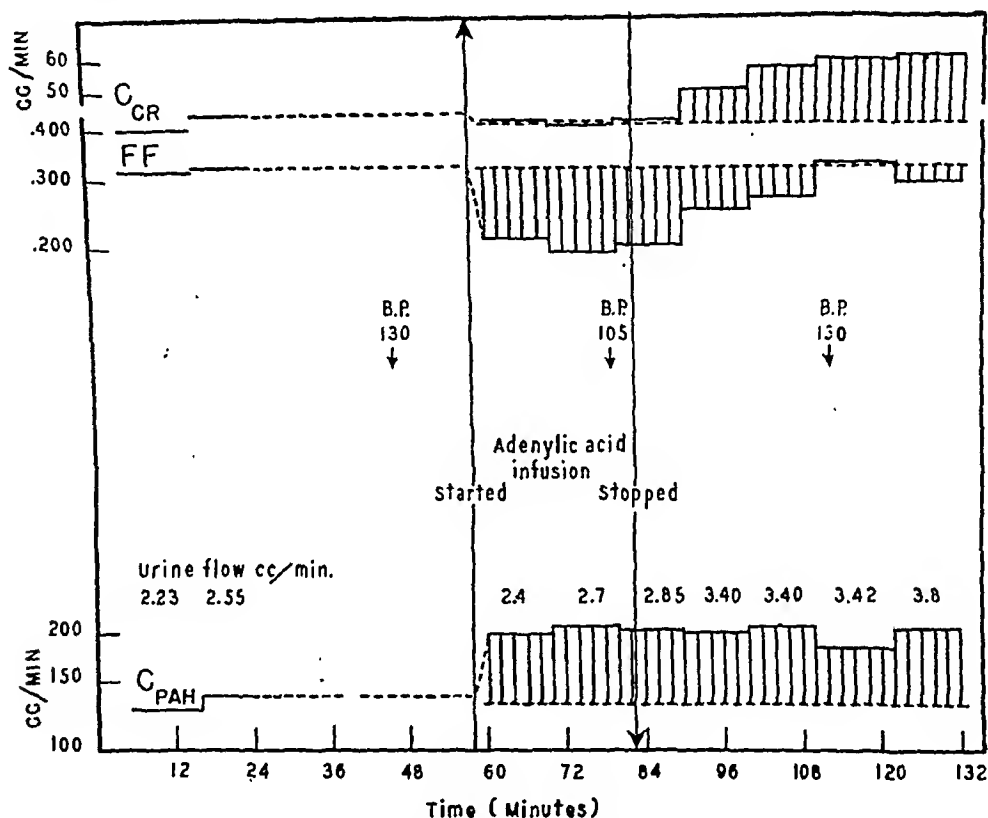


Fig. 3. EFFECTS OF THE INTRAVENOUS INFUSION of 1.10 mgm./kgm./min. of yeast adenylic acid into an 8.8 kgm. dog for 23.5 minutes (total dose = 25.9 mgm./kgm.) on filtration rate, renal plasma flow, and filtration fraction, all plotted on logarithmic ordinates, and mean arterial blood pressure and urine flow.

dilatation produced by adenine derivatives, but our data suggest that the renal arterioles may be more sensitive to such vasodilators than are the extra-renal arterioles.

Figure 4 shows an individual experiment in which 3.26 mgm/kgm./min. of yeast adenylic acid were infused over a period of 12 minutes, making a total of 39.2 mgm/kgm. infused. During infusion, blood pressure dropped from 115 to 40 mm. Hg, the filtration rate to 6 and plasma flow to 7 per cent of the pre-infusion level. Immediately after stopping the infusion, blood pressure and filtration returned to normal, while plasma flow increased to 168 per cent of control and gradually returned to normal after about 100 minutes. Figure 3 shows that infusion of a

smaller dose of adenylic acid (1.10 mgm./kgm./min. for 23.5 minutes, making a total of 25.9 mgm./kgm. infused) can cause very little drop in blood pressure and no change

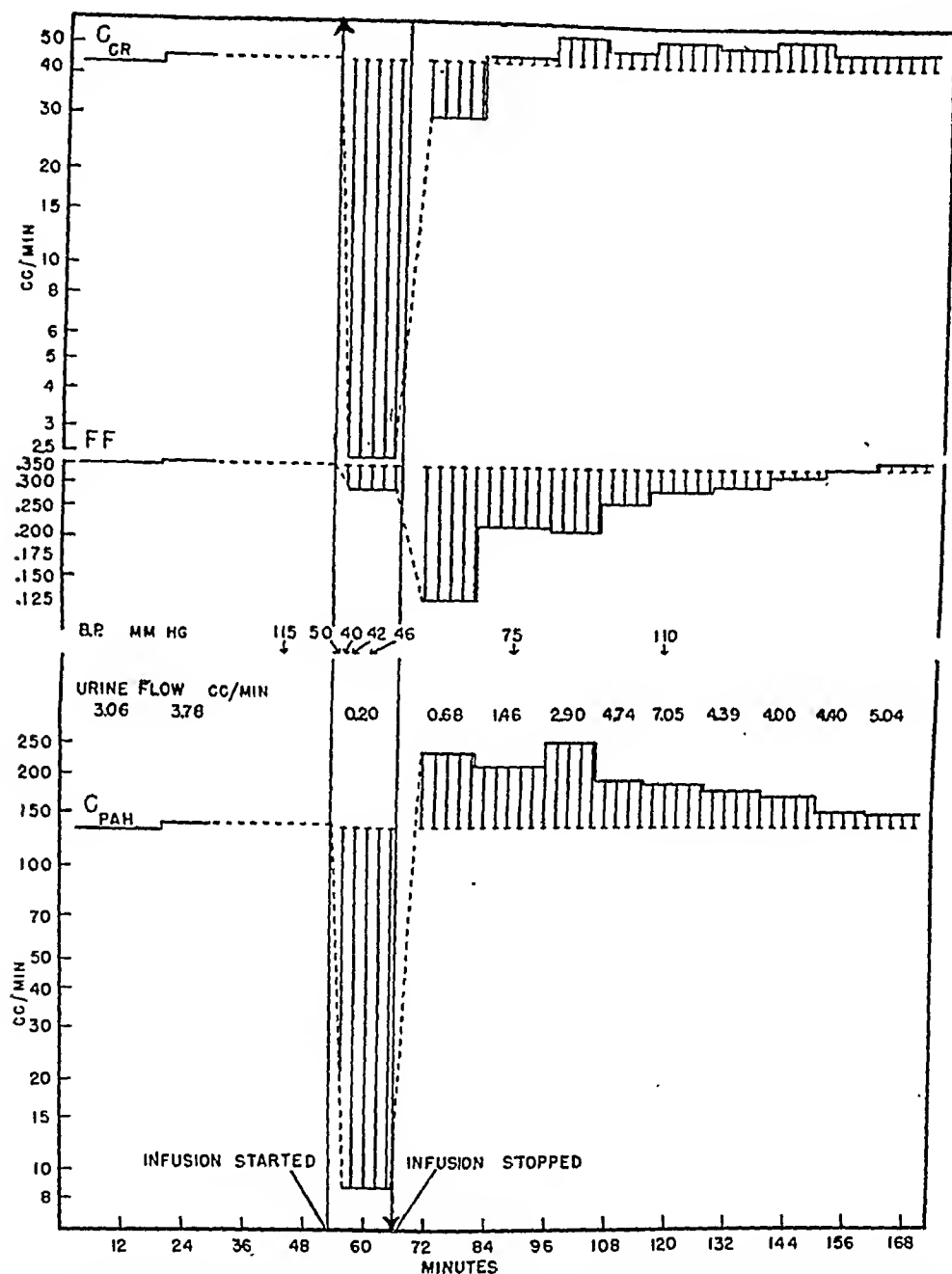


Fig. 4. EFFECTS OF THE INTRAVENOUS INFUSION OF 3.26 mgm./kgm./min. of yeast adenylic acid into a 22.8 kgm. dog for 12 minutes (total dose = 39.2 mgm./kgm.) on filtration rate, renal plasma flow, and filtration fraction, all plotted on logarithmic ordinates, and mean arterial blood pressure and urine flow.

in filtration rate and yet result in an increase in plasma flow to 155 per cent of control during the infusion. Termination of the infusion brought the blood pressure back

to the control level immediately, gradually raised filtration rate to 121 per cent of control, but did not alter plasma flow from its infusion level, even after 50 minutes at which time the experiment was terminated. Figure 2 illustrates the effects of infusion of 1.50 mgm./kgm./min. of yeast adenylic acid for 35 minutes, giving a total of 52.7 mgm./kgm. infused. Here a moderate drop in blood pressure occurred during infusion from 160 to 95 mm. Hg, filtration to 58 per cent of normal, while renal plasma flow increased to 116 per cent of control. On cessation of the infusion, filtration fraction returned to control, while plasma flow increased to 146 per cent of control and persisted for 85 minutes at which time the experiment was terminated.

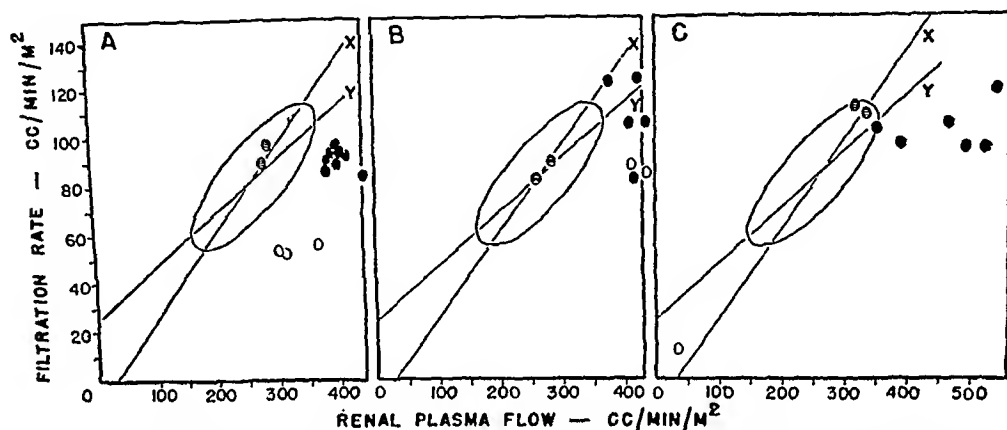


Fig. 5. RELATION OF FILTRATION RATE TO RENAL PLASMA FLOW during and after the intravenous infusion of adenylic acid or adenosine superimposed upon a control background based statistically upon this relation in 75 normal dogs (11). The ellipse embraces 70% of the points in the control series and corresponds roughly to a distance of ± 1 standard deviation from the mean on a linear scale for one function. X and Y are lines of regression based on the control series. Cross-hatched circles indicate pre-infusion (control) values; open circles, infusion values; closed circles, post-infusion values. A. Experiment illustrated in figure 2. B. Experiment illustrated in figure 3. C. Infusion of 3.17 mgm./kgm./min. of yeast adenosine in a 13.5 kgm. dog for 21 minutes (total dose = 66.6 mgm./kgm.).

The alterations in renal blood flow during and after infusion of these adenine derivatives are apparently statistically significant as revealed in figure 5 which shows that PAH clearance values for individual urine collection periods (solid symbols) in three experiments fall well outside the ellipse embracing 70 per cent of control filtration and flow values determined in 75 normal conscious dogs and reported in a previous paper (11).

Of particular interest is the rapid disappearance of adenylic acid from the blood after stoppage of the infusion of this compound. In conjunction with Kalckar, blood levels of both yeast and muscle preparations of this compound were determined in two experiments and the results are reported in some detail elsewhere (17). In one, 17.8 kgm. dog (no. 5), 26.8 mgm./min. of yeast adenylic acid were infused over a period of 35 minutes, giving a total of 938 mgm. infused. The pre-infusion plasma level of this compound was 0.25 mgm. per cent. Five minutes after starting the infusion (134 mgm. already infused) the level was 8.0 mgm. per cent. Eight minutes

after cessation of the infusion, the level had dropped to 4.7; 59 minutes after, to 1.0 mgm. per cent. In a similar experiment 214 mgm. of muscle adenylic acid was infused in a 15.8 kgm. dog (*no. 75*) over a period of eight minutes at the rate of 26.8 mgm/min. The pre-infusion plasma concentration of this compound was 0.2; 5 minutes after starting the infusion (147 mgm. already infused), 0.9; and 2 minutes after stopping the infusion, 0.25 mg. per cent. It is apparent that the muscle adenylic acid disappeared from the blood more quickly than the yeast preparation. This disappearance is attributed (17) to penetration into the cells and/or destruction by muscle and other cells, not solely to diffusion into the tissue fluid space.

Results qualitatively similar to those described above for yeast and muscle adenylic acid were obtained with yeast adenosine and sodium adenosinetriphosphate (table 1, fig. 1).

No significant correlation appears to exist between the above alterations of renal functions and either the rate of infusion or total amount of the experimental compound infused, when expressed on an absolute or on a kilogram basis (table 1).

However, it must be pointed out that the interrelationship of the above factors was not studied in the same animal, but in different animals. This may account for the poor correlation. One exception to this is *dog 75* infused with various amounts of muscle adenylic acid at various rates. The infusion of 232 mgm. at 18.5 mgm/kgm./min. resulted in a greater drop in filtration and renal plasma flow during the infusion, and a greater increase in flow in the post-infusion phase than did the infusion of practically the same total amount at 8.0 mgm/kgm./min.

A comparison of the five adenine derivatives with regard to their effects upon blood pressure or renal functions is not warranted in this paper because the data are too few to draw any conclusions.

Since pyrogenic substances produce renal hyperemia, it seemed necessary to eliminate an increased body temperature as a possible cause of the increased renal plasma flow obtained with these adenine derivatives. Green and Stoner (18) found an increased oral temperature 45 minutes after the intravenous administration of from 0.25 to 1.78 mgm/kgm. of the magnesium salt of ATP in three normal male subjects. No significant increase in rectal temperature which might result in hyperemia occurred in any of the dogs infused in the present investigation with any of the adenine derivatives in amounts ranging from 8.1 to 98.5 mgm/kgm.

Green and Stoner (18) report a diuresis in their cases mentioned in the previous paragraph. A moderate to marked diuresis was consistently observed in the present study in the post-infusion period. It occurred whether urine flow during the previous infusion period was decreased or not. Such a diuresis was not due to the volume of isotonic saline used as the vehicle for the infusion of adenylic acid, but to the dissolved experimental compound itself, for infusions of isotonic saline alone in comparable volumes and at comparable rates were not followed by a diuresis nor by an increase in either filtration rate or renal plasma flow, which one would expect if the infusion were markedly increasing the plasma volume (19). Consequently, the alterations in urine flow and renal plasma flow herein demonstrated must be due to the experimental compounds themselves.

No attempt has been made in this investigation to determine conclusively the

mode of action of adenylic acid, whether it acts directly upon the renal arterioles or secondarily through the splanchnic vasoconstrictor or other nerve fibers to the kidney. Since these adenine derivatives alter the tone of blood vessels in a perfused heart-lung-kidney preparation (16), and since the tone of the efferent arteriole can be altered by adrenaline and pyrogenic substances which appear to act directly upon the blood vessels (2), the authors favor the former explanation. Furthermore, the post-infusion hyperemia can hardly be attributed to a compensatory systemic reflex adjustment to the hypotensive state which occurs during the infusion phase, for such compensation would be expected to take the form of a vasoconstriction with a decreased renal plasma flow instead of a renal vasodilatation with a hyperemia.

The probable clinical significance of a renal hyperemia following the infusion of the adenine compounds cannot be estimated or properly appraised at this time. Since these compounds are general vasodilators, decreasing peripheral resistance to blood flow and thus causing a decreased blood pressure to shock levels, their use in the clinic to dilate renal blood vessels must be accompanied by the injection of some general vasoconstrictor. It is the hope that such a procedure might offset the extrarenal vasodilatation and prevent the decrease in mean arterial blood pressure, yet not act upon the renal arterioles. Ephedrine has been suggested (20) as such a vasoconstrictor substance, since it causes extrarenal vasoconstriction and raises blood pressure, but does not markedly alter renal blood flow (21). So far, the pyrogenic substances have been used with some reservation, with the aid of an antipyretic such as amidopyrine, to produce renal vasodilatation in human hypertension (22). It is obvious that these pyrogenic substances entail some risk due to their nature. Whether or not the correct combination of ephedrine and adenylic acid or some other adenine derivative will be the future renal vasodilator of choice for production of a much needed renal hyperemia in eclampsia, pre-eclampsia, hypertension and other conditions involving renal vasoconstriction must await further investigation.

SUMMARY AND CONCLUSIONS

1. The effects of the intravenous infusion of yeast and muscle adenylic acid, yeast adenosine or sodium adenosinetriphosphate upon glomerular filtration rate and effective renal plasma flow have been examined in 16 experiments in 10 conscious dogs.

2. The effects of yeast or muscle adenylic acid upon these renal functions fall into two phases *a*) the infusion phase and *b*) the post-infusion phase.

3. The infusion phase consists of a reduction in filtration rate ranging from 10 to 100 per cent, depending upon the degree of reduction in blood pressure. If filtration is between 65 and 100 per cent of the pre-infusion control, renal plasma flow remains constant or increases to as much as 180 per cent of control. However, if filtration drops to below 50 per cent of control, plasma flow decreases, to zero in some instances. In every case, the filtration fraction decreases. It is concluded that a combination of general arteriolar dilatation, afferent glomerular arteriolar constriction and efferent glomerular arteriolar dilatation accounts for these effects.

4. The post-infusion phase begins immediately after stopping the acid infusion. It consists of an immediate return of blood pressure and filtration rate to or near the

pre-infusion control level and, in every instance, a hyperemia, ranging from 111 to 240 per cent of control and lasting for as long as 100 minutes. Consequently, the filtration fraction remains reduced, indicating dilatation principally of the efferent glomerular arteriole and, to some degree, of the afferent vessels.

5. Qualitatively similar results are obtainable with yeast adenosine or sodium adenosinetriphosphate.

6. Under the experimental conditions of this investigation, no significant correlation could be established between the alterations of renal function during the infusion and post-infusion phases and the rate of infusion or total amount of the particular compound infused.

The authors wish to express their appreciation to Dr. Homer W. Smith, New York University, College of Medicine, for guidance and helpful criticism during the problem; to Dr. D. D. Van Slyke, Rockefeller Institute for Medical Research, for providing funds for the purchase of the muscle adenylic acid; to Dr. H. M. Kalckar, presently at the University of Copenhagen, Denmark, and to Dr. R. M. Archibald, Rockefeller Institute for Medical Research for analysis of plasma adenylic acid; to Dr. S. Ochoa, New York University, College of Medicine and to Dr. H. M. Kalckar for advice on the purification of the barium adenosinetriphosphate solutions; to Dr. G. H. Hitchings, The Wellcome Research Laboratories, Tuckahoe, New York, for analysis of this solution; and to Mr. Anthony Brittis, Mr. Gerald Weinberger, Miss Christine Waples, Miss A. Newman and Mrs. Louise Buchanan for able technical assistance during the course of this study.

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STATISTICAL ANALYSIS OF FILTRATION RATE AND EFFECTIVE RENAL PLASMA FLOW RELATED TO WEIGHT AND SURFACE AREA IN DOGS

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A STATISTICAL analysis of renal plasma flow and filtration rate in the normal dog, based upon a large series of observations similar to that available for man (1, 2), has not hitherto been published. Since numerous investigators are using the dog in studying the effects of drugs, hormones and nervous factors upon renal hemodynamics, control data on glomerular filtration rate and effective renal plasma flow obtained from time to time in our laboratories by various investigators² on 75 normal, trained, female dogs have been collated and are herein analyzed. The data serve to describe the normal range of each of these renal functions, relative to both body weight and surface area, in this species. The statistics, referred to body surface area, are compared with the corresponding statistics in man.

EXPERIMENTAL PROCEDURE

All dogs used in this analysis were females, trained to lie loosely restrained on a well padded animal board. In every instance the dogs were in the postabsorptive state and, in most instances, they were well hydrated before each clearance observation, thus obviating variations in renal plasma flow and filtration rate due to dietary intake of protein or poor state of hydration (3).

Surface area was calculated in most instances from the length-weight formula of Cowgill and Drabkin (4) as follows:

$$S.A. = \frac{2.268W^{0.37} \times L}{10,000}$$

where *S.A.* is surface area in square meters, *W* is the body weight in grams and *L* is the length in centimeters from nose to anus, measured along the belly. In some cases, the belly length was unavailable and the Meeh-Rubner weight formula was used:

$$S.A. = \frac{11.2W^{0.667}}{10,000}$$

The dogs ranged in surface area from 0.353 to 0.880 m²; in body weight, from 5.6 to 19.5 kgm.

Received for publication March 6, 1948.

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Glomerular filtration rate was measured by the creatinine clearance (2) and effective renal plasma flow by the clearance of p-aminohippuric acid (PAH) (5). These clearances were determined simultaneously. Plasmas were precipitated for creatinine and PAH determinations by the cadmium sulfate method of Fujita and Iwatake (6) and the creatinine concentrations of the plasma filtrate and diluted urines were determined by the method of Folin and Wu (7). PAH was determined by the method of Smith, Finkelstein, Aliminos, Crawford and Graber (5). All colorimetric determinations were made in duplicate and read on an Evelyn photoelectric colorimeter or a Coleman Junior Spectrophotometer. Diuresis was produced either by the administration of 50 cc/kgm. of water by stomach tube 30 minutes before U_0 , or by the intravenous infusion of a 10 per cent mannitol solution. Plasma levels of both creatinine and PAH were obtained by suitable priming injections into the jugular vein or infusion tubing 10 minutes before U_0 and maintained by constant intravenous infusion into either a marginal ear vein or a leg vein by a gravity, mercury-drip pressure apparatus. Urine was collected by an indwelling bladder catheter and blood was withdrawn periodically from the jugular vein through a 19-gauge needle.

RESULTS AND DISCUSSION

Average values for filtration rate and effective renal plasma flow in the 75 dogs are given in table 1, where the data are related both to body weight and body surface area. The results of statistical analysis of these data appear in table 2. The relationship between these two kidney functions is expressed graphically in figures 1 and 2. The ellipse appearing in each of these figures represents the area of a scatter diagram within which we may expect 70 per cent of the observations to fall by chance alone, assuming normal bivariant distribution.³ The ellipse corresponds roughly to the range $\pm 1\sigma$ on a linear scale for one function. This convenient graphic method of expression was first used in renal physiology by Goldring, Chasis, Ranges and Smith (1) and Smith (2) in the analysis of the same renal functions in man. The data which we have quoted on normal man (1, 2) have been related to body surface

³ The area of a scatter diagram within which we may theoretically expect 70 per cent of the observations to fall by chance alone is an ellipse formed by the equation

$$\chi^2 = \left(\frac{x^2}{\sigma_x^2} - \frac{2rxy}{\sigma_x\sigma_y} + \frac{y^2}{\sigma_y^2} \right) \frac{1}{1-r^2}$$

where χ^2 is taken from recorded χ^2 tables (8) and is determined by P , the proportion of observations which may be expected to fall outside the ellipse by chance alone, and by n , the degrees of freedom in the system. Here P is 0.30 (i.e., 70 per cent within the ellipse) and $n = 2$; hence, $\chi^2 = 2.408$. In the above equation x is the distance from mx along the x axis, y is the distance from my along the y axis, σ_x and σ_y are the standard deviations of the distribution in the x and y direction, respectively, and r is the coefficient of correlation. x is determined for various values of y by resolving the above equation in the quadratic.

$$x = \frac{-ay}{2} \pm \sqrt{\left(\frac{a^2}{4} - b\right)y^2 - c} \quad \text{and writing}$$

$$a = -\frac{2r\sigma_x}{\sigma_y}, \quad b = \frac{\sigma_x^2}{\sigma_y^2}, \quad \text{and} \quad c = -(1-r^2)\chi^2\sigma_x^2.$$

An ellipse on a scatter diagram within which 70 per cent of the observations may be expected to fall by chance corresponds roughly to a distance from -1σ to $+1\sigma$ on a linear scale for one variable (68 per cent of the observations). Pp. 637 and 638 (1).

TABLE 1. FILTRATION RATE, EFFECTIVE RENAL PLASMA FLOW AND FILTRATION FRACTION IN 75 NORMAL UNANESTHETIZED FEMALE DOGS, EXPRESSED AS ACTUAL VALUES AND RELATED TO BODY WEIGHT AND BODY SURFACE AREA

DOG	NO. OF OBSERV.	BODY WEIGHT (KGM.)	BODY SURFACE AREA (M ²)	FILTRATION RATE (C_{CR}) IN CC./MIN.			EFFECTIVE RENAL PLASMA FLOW (C_{PAH}) IN CC./MIN.			FILTRATION FRACTION C_{CR}/C_{PAH}
				Actual value	Per kgm. body wt.	Per m ² body S.A.	Actual value	Per kgm. body wt.	Per m ² body S.A.	
1	1	15.0	.683	55.8	3.72	81.7	197	13.10	289	.283
2	3	7.2	.450	42.1	5.85	82.9	107	14.90	247	.335
3	5	15.0	.683	54.3	3.62	79.5	163	10.90	239	.339
4	2	16.0	.715	59.0	3.69	82.5	200	12.50	279	.295
5	13	17.8	.765	66.9	3.75	86.2	215	12.08	281	.306
6	1	11.0	.620	34.0	3.09	54.6	117	10.62	188	.290
7	4	13.5	.638	58.6	4.35	91.8	173	12.80	271	.353
8	6	9.3	.496	43.5	4.69	87.6	124	13.33	249	.335
9	2	12.0	.588	39.3	3.27	66.7	119	9.90	203	.348
10	7	18.0	.775	70.0	3.89	90.5	227	12.60	294	.305
11	8	15.2	.691	73.0	4.81	105.4	228	15.00	329	.309
12	10	13.5	.638	68.6	5.08	107.7	239	17.70	375	.311
13	6	10.9	.540	50.5	4.62	93.4	163	14.95	302	.307
14	8	19.5	.880	85.3	4.38	96.9	298	15.30	338	.296
15	5	19.5	.740	90.0	4.61	121.4	293	15.05	396	.284
16	4	8.8	.478	50.5	5.74	105.8	155	17.60	325	.325
17	1	10.0	.522	64.0	6.40	122.5	210	21.00	403	.304
18	3	11.0	.556	37.1	3.37	66.6	138	12.53	248	.284
19	1	12.0	.588	59.8	4.98	101.6	197	16.40	336	.303
20	4	18.0	.775	70.6	3.92	91.2	266	14.80	343	.306
21	3	12.0	.588	57.0	4.75	97.0	174	14.50	296	.327
22	6	17.5	.756	73.0	4.17	96.6	240	13.70	318	.303
23	3	18.0	.775	85.2	4.74	110.0	267	14.85	344	.330
24	1	6.5	.392	32.4	5.00	82.7	104	16.00	265	.312
25	5	9.0	.550	48.9	5.44	88.8	192	20.20	331	.269
26	1	9.6	.460	40.4	4.20	88.0	164	17.10	356	.247
27	2	8.5	.478	31.5	3.72	65.9	90	10.58	187	.352
28	3	6.8	.442	30.1	4.43	68.2	95	13.90	214	.319
29	4	10.3	.580	50.0	4.86	86.3	173	16.80	298	.289
30	3	7.8	.495	25.0	3.22	50.5	96	12.30	193	.262
31	3	8.2	.505	40.5	4.94	80.1	126	15.32	249	.322
32	7	6.7	.396	27.0	4.02	68.1	116	17.30	294	.232
33	1	11.7	.588	25.2	2.15	42.8	102	8.75	174	.246
34	10	13.3	.630	51.0	3.83	80.8	177	13.30	281	.287
35	3	14.5	.660	72.6	5.00	110.0	284	19.60	430	.256
36	2	18.6	.800	69.0	3.71	86.1	234	12.55	293	.294
37	3	13.8	.650	44.0	3.19	67.7	162	11.75	249	.272
38	2	14.5	.680	55.5	3.83	81.7	169	11.65	249	.328
39	7	7.0	.410	34.4	4.92	84.0	126	18.00	307	.278
40	19	17.0	.867	62.7	3.69	62.7	217	12.75	250	.289
41	12	14.5	.670	42.5	2.93	63.3	166	11.45	248	.255
42	1	9.3	.592	77.4	8.32	131.0	171	18.40	290	.452
43	1	12.8	.705	43.4	3.39	61.6	120	9.40	170	.363

TABLE 1.—Continued

DOG	NO. OF OBSERV.	BODY WEIGHT (KGM.)	BODY SURFACE AREA (M ²)	FILTRATION RATE (C_{CR}) IN CC/MIN.			EFFECTIVE RENAL PLASMA FLOW (C_{PAH}) IN CC/MIN.			FILTRATION FRACTION C_{CR}/C_{PAH}
				Actual ¹ value	Per kgm. body wt.	Per m ² body S.A.	Actual ¹ value	Per kgm. body wt.	Per m ² body S.A.	
44	5	15.1	.690	76.5	5.06	110.8	200	13.20	290	.382
45	4	13.8	.645	86.0	6.22	133.2	243	17.60	376	.354
46	1	13.8	.645	71.2	5.17	110.0	199	14.45	309	.356
47	1	11.4	.562	35.6	3.13	63.5	94	8.24	167	.380
48	4	11.8	.574	45.6	3.87	79.6	158	13.40	275	.289
49	1	9.3	.500	26.3	2.84	52.5	101	10.90	202	.259
50	1	8.7	.478	27.4	3.16	57.2	83	9.60	173	.331
51	4	18.2	.812	86.0	4.73	106.0	309	17.00	380	.279
52	1	13.4	.630	53.6	4.00	85.0	238	17.70	378	.225
53	5	11.2	.690	51.5	4.60	74.9	129	11.52	187	.400
54	2	8.1	.505	55.0	6.80	109.0	182	22.43	332	.329
55	1	11.7	.610	51.9	4.43	85.0	117	10.00	192	.443
56	4	10.6	.615	62.5	5.90	101.4	186	17.50	302	.336
57	3	14.7	.675	76.4	5.20	113.2	270	18.40	400	.283
58	1	11.8	.588	57.2	4.85	97.5	156	13.20	265	.368
59	3	8.5	.508	25.4	3.00	49.9	71	8.40	139	.359
60	2	12.0	.725	50.0	4.16	69.0	105	8.75	145	.475
61	1	5.8	.362	24.4	4.41	67.4	75	13.05	207	.325
62	1	6.0	.371	22.5	3.74	60.7	66	10.90	177	.343
63	1	5.6	.353	21.7	3.95	61.5	57	10.10	162	.380
64	2	11.83	.578	47.9	4.04	82.8	159	13.40	275	.302
65	2	12.5	.602	61.2	4.90	101.7	211	16.85	351	.290
66	2	12.3	.602	39.8	3.23	66.2	111	9.00	185	.357
67	2	12.1	.597	42.6	3.62	71.4	129	10.70	217	.329
68	2	12.0	.590	33.4	2.78	60.3	97	8.05	168	.359
69	1	10.1	.522	44.0	4.35	84.3	131	12.96	251	.336
70	1	12.9	.618	47.3	3.66	76.4	123	9.54	199	.384
71	1	15.0	.690	59.0	3.93	85.4	125	8.30	181	.472
72	1	16.0	.715	44.2	2.76	61.6	162	10.10	227	.271
73	1	18.0	.775	73.5	4.07	94.8	200	11.10	258	.367
74	1	18.0	.775	72.2	4.02	93.1	199	11.05	257	.362
75	1	12.5	.607	63.2	5.05	104.0	156	12.47	257	.404

¹ Each value is the average of separate observations, the number of which is indicated in column 2. Each observation in turn is an average of from 2 to 3 individual urine collection periods of approximately 10 minutes each.

area and the statistical analysis is included for comparison with the dog (fig. 3, table 2).

In both dog and man, the correlation coefficient between renal plasma flow and filtration rate referred to surface area is good, i.e., in both species a high filtration rate is usually associated with a high renal plasma flow, and a low filtration rate with a low renal plasma flow. The difference in correlation coefficient in dog (0.79) and man (0.82) is probably not significant. It is interesting to note that the lines of regression in each species straddle the origin (figs. 1 and 3), indicating that the

physiological zero on the scale of one function is in the neighborhood of zero on the scale of the other function.

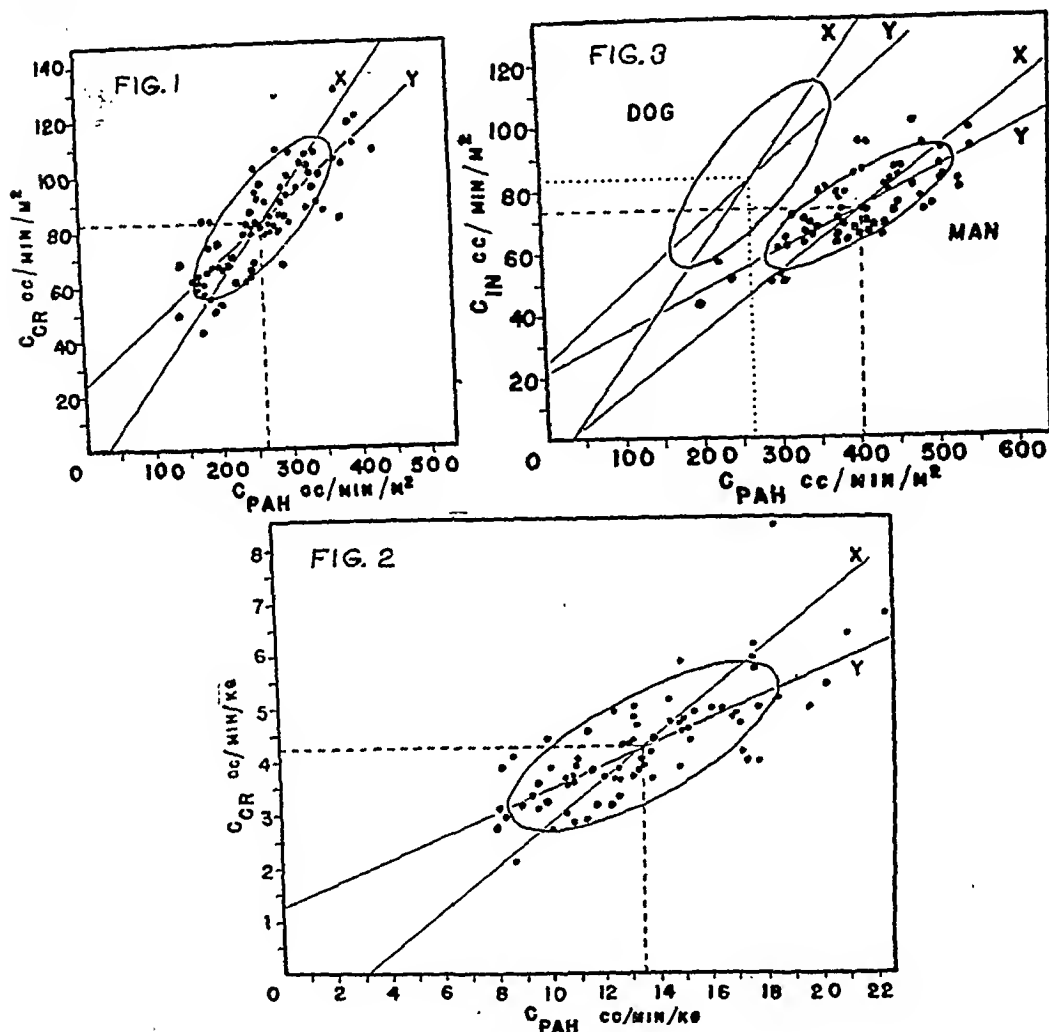


Fig. 1. COMPARISON OF FILTRATION RATE (C_{CR}) AND RENAL PLASMA FLOW (C_{PAH}) AS RELATED TO BODY SURFACE AREA IN 75 NORMAL DOGS. Each point represents a different dog and is the average of from one to 19 clearance observations (table 1). Dashed lines are the means for each function. The ellipse delimits the area of a scatter diagram within which 70 per cent of the points may be expected to fall by chance alone, assuming normal bivariate distribution. X represents the regression of x (renal plasma flow) on y (filtration rate), i.e., the variation along the x axis for a given value of y ; and vice versa.

Fig. 2. COMPARISON OF FILTRATION RATE AND RENAL PLASMA FLOW, AS RELATED TO BODY WEIGHT, IN 75 NORMAL DOGS. See legend of figure 1 for details.

Fig. 3. COMPARISON OF FILTRATION RATE AND RENAL PLASMA FLOW, AS RELATED TO BODY SURFACE AREA, IN DOG AND MAN. The ellipse and lines of regression for the dog are transposed from figure 1. Those for man are calculated from data in the literature (2) and are summarized in table 2 of this paper.

Inspection of table 2 and figure 3 reveals certain quantitative differences between dog and man with regard to filtration and renal plasma flow. Per square meter of

body surface area, the dog has a greater mean filtration rate than man (84.4 vs. 75) but a smaller mean renal plasma flow (266 vs. 403), thus giving a higher mean filtration fraction in the dog (32 per cent) than in man (19 per cent).⁴ The coefficient

TABLE 2. STATISTICAL ANALYSIS OF FILTRATION RATE AND EFFECTIVE RENAL PLASMA FLOW RELATED TO BODY WEIGHT AND BODY SURFACE AREA IN THE NORMAL DOG. COMPARISON WITH SIMILAR STATISTICAL ANALYSIS IN NORMAL MAN¹

STATISTIC	DOG (PRESENT STUDY)					MAN (1, 2)		
	C _{CR} (cc/min.)		C _{PAH} (cc/min.)		C _{CR} / C _{PAH}	C _{IN} (cc/ min.)	C _{PAH} (cc/min.)	C _{IN} / C _{PAH}
	per m ² body S.A.	per kgm. body wt.	per m ² body S.A.	per kgm. body wt.		per m ² body S.A.	per m ² body S.A.	
Number of cases <i>n</i>	75					61		
Mean <i>m</i>	84.4	4.29	266	13.51	0.317	75.0	403	0.186
Range	43-133	2.15-8.32	139-430	8.05-22.43	225-475	51.4-103.2	198-546	151-257
Standard deviation <i>σ</i>	19.1	1.01	66	3.26	0.052	12.7	78.5	0.0244
Coefficient of varia- tion 100 $\frac{\sigma}{m}$	22.6	23.6	24.8	24.1	16.4	16.9	19.5	12.8
Standard error $\sigma_m = \frac{\sigma}{\sqrt{n}}$	2.2	0.115	7.6	.375	0.006	1.63	10.1	0.0031
Coefficient of corre- lation <i>r</i>	0.79	0.73				0.82		

Number of dogs = 75.
Number of simultaneous clearance observations = 258.
Number of individual 10-minute urine collection periods = 516 to 774.

Number of subjects = 61.
Number of simultaneous clearance observations = 99.
Number of individual 10-15 minute urine collection periods = 198 to 297.

¹ Data on man are from 'combined series, normal men', pp. 96-97 (2).

of variation of filtration rate, renal plasma flow and filtration fraction in the dog is slightly higher than in man (table 2), indicating that renal function in the dog is more labile than in man.

When filtration rate and renal plasma flow are related to body weight in the dog, the correlation is essentially as good as when these functions are related to surface

⁴ This is of course true whether or not the two variables are simultaneously corrected to body surface area.

area, and the coefficients of variation are practically the same. This would indicate that in dogs of the weight range studied here, body weight is as good a standard of reference as body surface area for these renal functions. However, studies on human infants suggest that the correlation with surface area would probably be better over a wider range in animal size (9).

SUMMARY

1. A statistical analysis is presented of filtration rate and effective renal plasma flow, as related to body weight and surface area in 75 normal, trained, female dogs.
2. The mean filtration rate in the 75 animals per sq.m. of body surface area is 84.4 cc. per minute, with a standard error of 2.2; the range of observations is from 43 to 133 cc. per minute. Expressed per kgm. of body weight these figures are: mean 4.29, standard error 0.115, range 2.15 to 8.32 cc. per minute.
3. The mean effective renal plasma flow in the 75 animals per sq.m. of body surface area is 266 cc. per minute, with a standard error of 7.6; the range of observations is from 139 to 430 cc. per minute. Expressed per kgm. of body weight these figures are: mean 13.5, standard error 0.375, range 8.05 to 22.4 cc. per minute.
4. The mean filtration fraction is 0.317, indicating that roughly 32 per cent of the plasma flowing through the glomerulus is filtered.
5. The coefficient of correlation between plasma flow and filtration rate is 0.79 when these are related to body surface area, and 0.73 when related to body weight, indicating a relatively good correlation regardless of the unit of body size.
6. An ellipse has been calculated to delimit the area of a scatter diagram of filtration rate plotted against renal plasma flow within which about 70 per cent of observations may be expected to fall if we assume that we have a normal bivariate distribution. This is equivalent roughly to a range of $\pm 1\sigma$ from the mean on a linear plot of either function.
7. The above statistics in the dog are compared with those in a combined series of normal men computed from data reported by other investigators.
8. The dog has a higher filtration rate than man, but a smaller effective renal plasma flow; therefore, the filtration fraction in the dog is higher than in man.

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COMPARISON OF THE ELECTROCARDIOGRAPHIC CHANGES PRODUCED BY HEATING AND COOLING EPICARDIAL AND ENDOCARDIAL SURFACES OF THE DOG VENTRICLE¹

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THERE is no agreement regarding the contribution made by subendocardial layers of the myocardium to the electrocardiogram. According to one hypothesis, the whole of the ventricular electrocardiographic complex develops from the sequential appearance and disappearance of local differences of potential between the cavity and the external surface of the heart.

Thus in the chest lead the R wave is supposed to develop as the wave of excitation travels outward from endocardium to epicardium beneath the chest electrode and terminate when the full thickness of the myocardium in this region is depolarized. Similarly, the T wave is thought to represent the recurrence of potential differences between endocardium and epicardium in the same area because of differences in the time and rate of repolarization. An upright T wave is said to indicate that the epicardial layers complete their repolarization earlier than the endocardial areas, while inversion of T is considered to have an opposite significance. Another hypothesis has been brought forward which derives the electrocardiogram as recorded from limb and chest leads from imbalances of potential caused by differences in the state of excitation and recovery of large segments of the heart without respect to their inner and outer lamellae. Thus in the standard limb leads of the dog, the electrocardiogram is considered to reflect the asynchronous excitation and recovery of large and relatively equal segments in the right and left ventricles. In the chest leads these segments are still large but distinctly unequal in size and bear reference primarily to the position of the exploring electrode rather than to the anatomical division of the heart (1). According to this view, the spread of a wave of excitation or of repolarization through the thickness of a ventricular wall is not detectable in the electrocardiogram (2).

Experiments in which endocardial and epicardial extrasystoles were elicited from a great number of positions in both ventricles by means of electrodes placed directly opposite each other on the epicardium and endocardium with each pair in the axis of the limb leads or directly beneath the chest electrode failed to reveal differences in configuration of the QRS complex between the endocardial and epicardial extrasystoles. These experiments made it improbable that waves of excitation traveling in either direction between the endocardium or epicardium are detectable in the standard body-surface leads, and highly unlikely that the R wave in the chest lead develops from the outward spread of the wave of excitation as it proceeds from the

Received for publication February 12, 1948.

¹ This work was supported by grants from the Cooper Fund, Faculty of Medicine, McGill University, and the Fluid Research Fund, Yale University School of Medicine.

endocardium to epicardium through the thickness of the myocardium beneath the chest electrode.

These results made it desirable to determine whether the T wave is in any way influenced in a comparable manner by the wave of recovery which might, depending upon differences in the relative rates of repolarization of epicardial and endocardial laminae, proceed from within outward or from without inward, and so produce in the chest leads inverted or upright T waves, respectively. The experiments now to be reported were carried out by the method first employed by Burdon-Sanderson and Page (3) of localized heating or cooling of restricted areas of both the endocardium and epicardium.

METHODS

The experiments reported here were carried out on 18 dogs, deeply anesthetized with nembutal and maintained on artificial respiration by endotracheal insufflation with oxygen. Full inflation of the lungs and practically full closure of the chest wall were maintained despite the ingress and egress of the fine rubber tubing carrying the cooling and warming fluid and the stem of the endocardial thermode. Curare was at times employed to suspend respiration motions. (Entocostin, Squibb, kindly supplied by E. R. Squibb and Co., through the courtesy of R. M. Stoddard.) Temperature changes were produced by means of a flat thermode applied to the external surface of the ventricles, through which water at an appropriate temperature could be circulated (4). In most instances the epicardial thermode was a hollow metallic disk, 1 cm. in diameter. The endocardial thermode consisted of a hollow plastic sleeve 1.0 cm. in diameter, carrying at its distal end a hollow brass tip 1.0 cm. in diameter and approximately 2.0 cm. long. Inlet and outlet tubes contained in the plastic sleeve supplied the tip with warm or cold water. Insertion and positioning of the endocardial thermode was facilitated by some flexibility of the plastic sleeve, which could then be maintained in the desired position by a supporting stem connected to the thermode by a universal joint. In a final model, an extension of one of the copper tubes supplying the brass tip served as an adjustable stem for the thermode. The over-all length of the thermode itself was 10 cm.

In applying the thermode to the endocardium adequate contact at the chosen location was maintained without excessive pressure. This precaution was particularly necessary to avoid stimulation of the heart while a portion of the ventricle was being heated or cooled since the thermal gradient between treated and untreated regions increased the susceptibility of the heart to the development of ventricular fibrillation.

Lead II and one of the precordial leads (CR, CF, CL, or CV) were recorded before and during the heating and cooling of corresponding regions of the epicardium and endocardium in both the right and left ventricles. Control records were taken only after the positioned thermode was warmed approximately to the temperature of the heart and a steady T-wave height established. Usually the chest electrode was placed immediately external to the thermode, but in some instances the chest electrode was placed over the apex of the heart while the thermode was in position at the base, or the exploring electrode was placed over the apex of the heart while the ther-

mode was in position at the base or the exploring electrode was placed on the opposite side of the chest from the thermode.

In all hearts studied, and particularly so in the smaller ones, the left ventricular cavity narrowed at the apex and the brass tip of the endocardial thermode often came in contact with the anterior, posterior or septal walls in addition to the wall directly under the position of the chest electrode, while at times the distal portion of the thermode came in fairly close contact with the septum near the base. Such undesirable contacts had to be avoided or minimized, for, as will be seen, different regions of the endocardium have a different influence upon a chest electrode fixed in any one position.

RESULTS

Whenever an area of the endocardium of either ventricle was heated or cooled, the effect upon the electrocardiogram recorded by an immediately external chest electrode was the same as when the overlying epicardium was heated or cooled (fig. 1-4). Warming the endocardium increased the amplitude of the T wave, while cooling diminished or inverted it; similar treatment of the epicardium had the same effect. Whenever the region treated (endocardial or epicardial) was sufficiently distant to the position of the chest electrode the direction of the T-wave changes evoked by warming or cooling was the opposite of that resulting from similar treatment of the region lying directly beneath the chest electrode.

In the left ventricular cavity it was found that such 'distal' effects were often produced by contact of the thermode with the septum. This made it necessary to keep the thermode well lateral to the septum when the effect of heating and cooling of the apex and lateral wall of the left ventricle was being studied in chest leads from the apical area.

The heart regions contributing to Lead III were also explored. Warming of either the epicardial or endocardial surface of the anterior right ventricle resulted in inversion of T_3 , while cooling either surface increased the height of T_3 (fig. 5, 6). Warming of either the epicardial or endocardial surface of the left posterior ventricle resulted in increased height of T_3 while cooling either surface decreased the height of T_3 . Treatment of the upper third of the endocardial surface of the interventricular septum facing the left ventricular cavity as well as the overlying epicardial area resulted in T-wave changes similar to those obtained from treatment of the right anterior ventricle.

The development of T-wave changes following heating or cooling of the endocardium appeared to be somewhat slower than that following epicardial treatment, and rarely reached the same magnitude. In each case, however, the development of T-wave change was progressive, and the maximum once obtained remained constant until the heating or cooling was discontinued.

DISCUSSION

The results obtained in these experiments on dogs' hearts indicate that one cannot differentiate between the contribution of the endocardial and the overlying epicardial layers in the formation of the T wave. Acceleration of the rate of repolari-

zation by heat produces sharply upright T waves in chest leads when either the endocardium or epicardium lying directly under the electrode is treated. Such T-wave changes have been observed in the early stages of infarction as well as during anoxia and in the light of these experiments must be explained on the basis of accelerated repolarization under the electrode, rather than on a difference in response of the endocardial and epicardial layers to the oxygen deficiency. Delay in the rate of repolarization by cooling produces inversion of the T wave in chest leads, whether endocardial or epicardial layers under the electrode are treated. Such T-wave

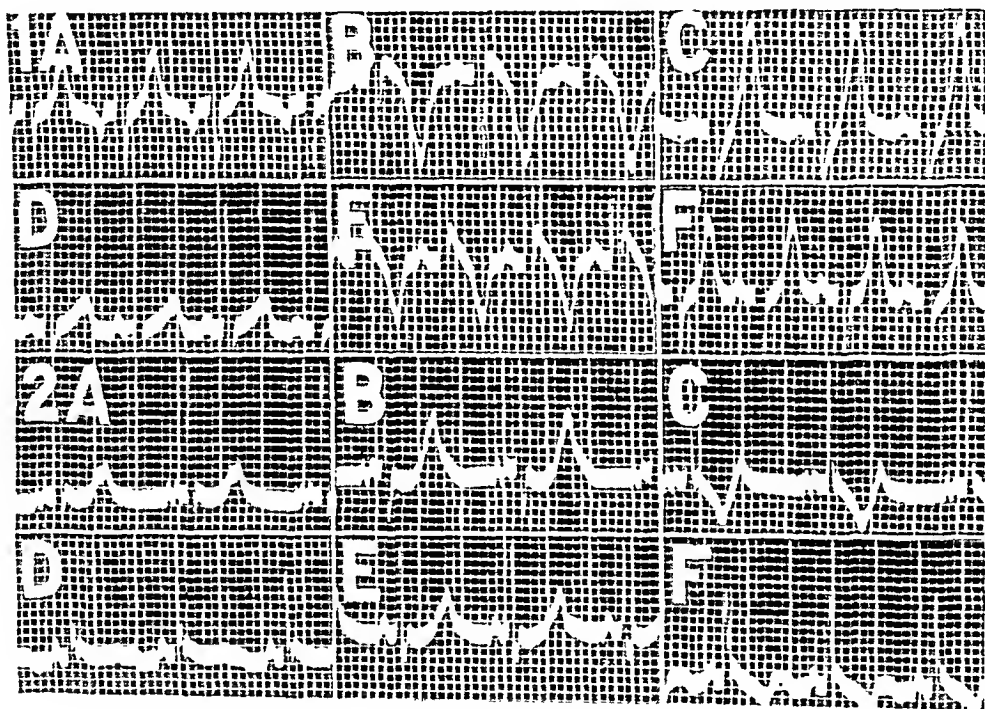


Fig. 1. April 28, 1947. C V LEAD WITH THE CHEST ELECTRODE ON THE RIGHT SIDE OF THE CHEST IMMEDIATELY EXTERNAL TO THE APEX OF THE RIGHT VENTRICLE. Heating and cooling applied to right apex; the region heated and cooled was therefore in the immediate vicinity of, i.e., 'proximal' to, the exploring electrode. A, B, C, outside, D, E, F, inside the ventricle. A, D, controls, with thermodes in place, B, E, cold; C, F, warm.

Fig. 2. April 29, 1947. C V LEAD WITH CHEST ELECTRODE OVER RIGHT APEX. Thermodes at right base, or in a distal area with respect to the chest electrode; effects are therefore reversed from fig. 1, but external and internal effects are the same. A, B, C, thermode outside, D, E, F, thermode inside the ventricle. A, D, control with thermodes in place; B, E, cooling; C, F, warming.

changes are characteristically found in the late stages of myocardial infarction and are to be explained by delay in repolarization which is well known to occur in this stage, and not on a difference in response of epicardial and underlying endocardial layers.

When thermal changes were produced at regions at a distance from the chest electrode, heating inverted the T wave while cooling increased the amplitude of the T wave, without regard to whether the thermal changes affected the epicardial or endocardial layers. Thus in the late stages of infarction an inverted T wave in a

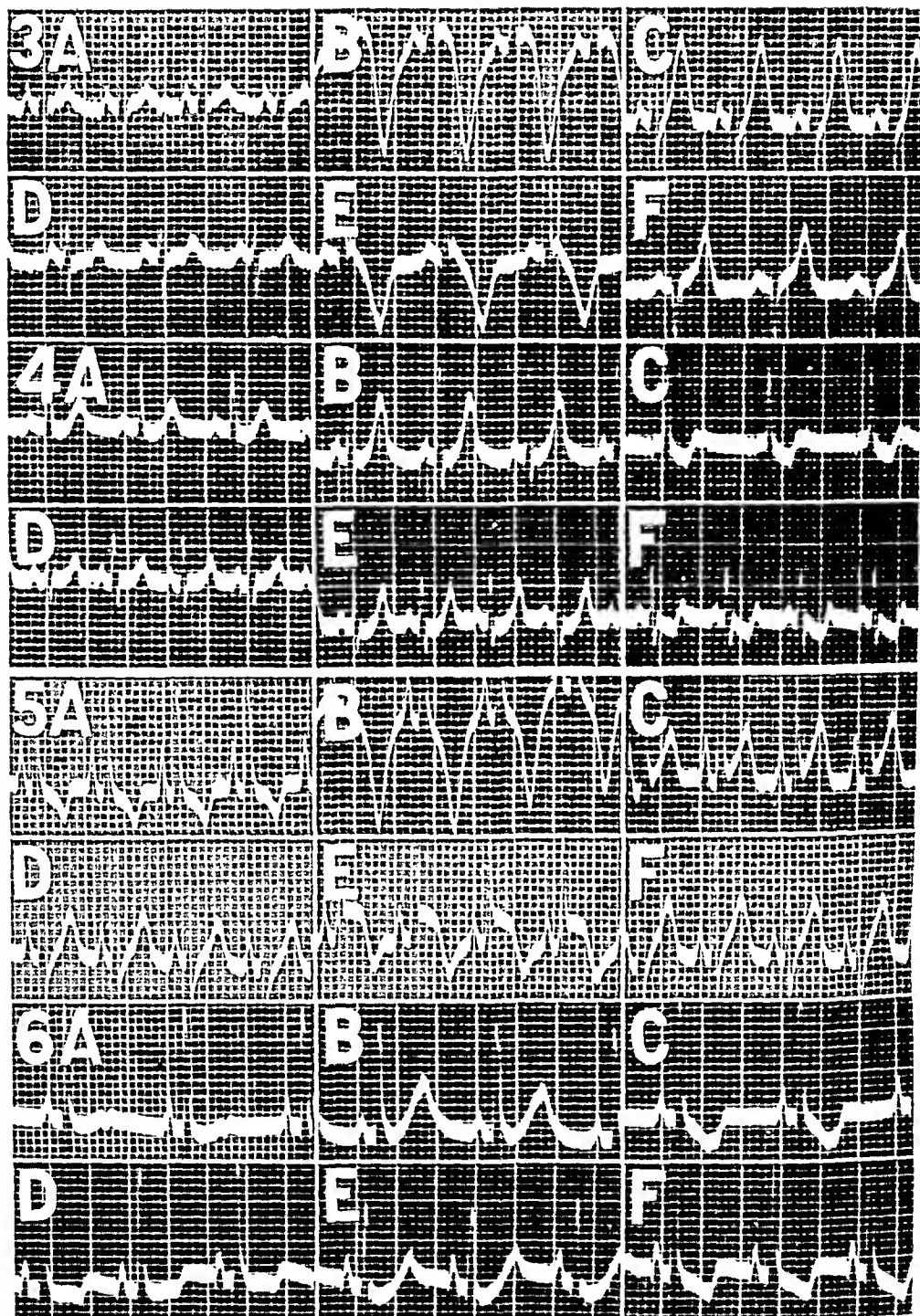


Fig. 3. November 25, 1946. C V LEAD WITH CHEST ELECTRODE IMMEDIATELY EXTERNAL TO LEFT APEX. Thermodes placed on left lateral wall at the apex, i.e., proximal to chest electrode. A, B, C thermode outside left ventricle; D, E, F, thermode inside left apex. A, D, control; B, E, cooling; C, F, warming

Fig. 4 April 29 1947. C V LEAD WITH CHEST ELECTRODE OVER LEFT APEX. Thermodes at the left base, i.e., distal to chest electrode; effects of warming and cooling are therefore the reverse of fig. 3. A, D, control, thermodes outside and inside, respectively. B, E, cooling; C, F, warming.

Fig. 5. December 4, 1946. LEAD III. Thermodes at the posterior wall of the left ventricle near the apex; A, B, C, inside; D, E, F, outside. A, D, control; B, E, cooling; C, F, warming. (The elevation of the S-T segment in E is not due to anoxia or interference with circulation, but to a reversible effect of the cold itself.)

Fig. 6. April 29, 1947. LEAD III. Thermodes at the anterior base of the right ventricle; A, B, C, outside; D, E, F, inside. A, D, control; B, E, cooling; C, F, warming.

chest lead indicates the presence of delayed repolarization in a region beneath the electrode, while an abnormally upright T wave indicates delayed repolarization in a region at a distance from the electrode.

In analyzing the T-wave changes in Lead III resulting from heating and cooling selected regions of the endocardium and overlying epicardium, it can be seen that both surfaces act in a similar manner. Slowed repolarization of the anterior right ventricle and of some septal portions of the left ventricle is reflected in an increase in the amplitude of T_z , but this wave becomes inverted when repolarization is slowed in the left posterior ventricle. Although the present studies were restricted for technical reasons to the anterior right and posterior left ventricles, there is no reason to doubt that the epicardial and endocardial surfaces of the posterior right and anterior left ventricles would behave in a similar manner.

These experiments lend no support to the assertion that an endocardial lesion which slows or hastens repolarization would produce alterations in the T wave in either the standard limb leads or in chest leads of a nature opposite to that which would develop were the immediately overlying epicardium involved. In chest leads there are epicardial areas near the precordial electrode whose electrical effects are oppositely directed to those of epicardial regions at a distance from the electrode and thus produce opposite effects on the T wave. Likewise, there are endocardial areas subjacent to the electrode whose electrical effects are oppositely directed to those of endocardial areas at a distance from the electrode. At any one given region of the heart, however, the contribution to the formation of the T wave of the inner and outer layers of the myocardium is similar. These experiments therefore confirm for the T wave the view previously expressed for the R wave (4) that the full thickness of the ventricular wall underneath the exploring body surface electrode acts electrically as a unit. Factors which alter the rate of repolarization in any part of it will affect the T wave in the same manner. The influence of the electrical events from the muscle mass proximally oriented toward the exploring electrode is balanced with the electrical events occurring in regions of the heart at a distance from the exploring electrode without regard to distinction between epicardial or endocardial layers. Both the QRS complex and the T wave are inscribed as the result of the electrical events occurring simultaneously in myocardial regions proximal and distal to the exploring electrode. The positions and magnitude of the proximal and distal zones differ for each position of the exploring electrode and therefore require experimental delimitation for each separate lead.

In the light of these experiments it is difficult to discuss the results recently reported by Byers, Toth and Ashman (5) after heating and cooling the endocardium of the right and left ventricle in the dog. Their injection of as much as 50 cc. of Ringer's solution in the arrested dog's ventricle would almost certainly affect the whole of the endocardium and make it impossible to differentiate between its various regions, which, as the present experiments demonstrate, is necessary, especially in the left ventricle. Here it has been shown that the base acts differently from the apex, which also acts differently from the septum. Only by comparing a region of epicardium and directly underlying endocardium in their response to heating and cooling can one determine whether the endocardial contribution is electrically of

opposite sign to that of the epicardium. Despite the limitations of their method, the results of these authors on heating and cooling the right ventricle confirm exactly those now reported. They observed that cooling the right ventricular endocardium increased the amplitude of T_3 , while warming reduced it. This is exactly what happens when a localized area in the epicardium of this ventricle is cooled. The findings in our experiments on heating and cooling the right ventricular endocardium are indistinguishable from the changes obtained in the electrocardiogram following similar treatment of the overlying epicardium.

It was noted that the effect upon the T wave of heating or cooling the endocardial surface was somewhat delayed when compared with similar treatment of the overlying epicardium. The explanation for this phenomenon cannot be given on the basis of the experiments reported here. One possibility is that with the rapid blood flow around the endocardial thermode it was not possible to achieve the same temperature differences as when the thermode was on the epicardium. Another possibility is that only when the heat or cold applied to the endocardium penetrates to the epicardial layers does the T-wave change begin to appear.

SUMMARY

In 18 dogs the influence on the T wave in the standard limb leads and precordial leads was studied, following warming and cooling of restricted areas of the endocardium and overlying epicardium of both the right and left ventricles. In all experiments the T-wave changes so produced were the same regardless of whether the endocardial or epicardial surfaces of a given region were warmed or cooled. Warming the endocardium and epicardium of the zone underneath the chest electrode (both in the right and in the left ventricle) produced sharp increase in the positivity of T. Conversely, cooling this area produced inverted T waves. Warming areas at a sufficient distance from the exploring electrode produced opposite effects, namely, inversion of T regardless of whether the endocardium or epicardium was treated.

In Lead III the effects of cooling or warming the endocardium in the right or left ventricle corresponded with results obtained from treatment of epicardial regions. Warming the anterior surface of the right ventricle endocardially or epicardially inverted the T wave while cooling increased it. Some septal and basal areas of the left ventricle give similar results. Cooling the posterior surface and apex of the left ventricle inverted the T wave whether the endocardium or epicardium was affected. Warming these regions elevated the T wave.

The experiments lend no support to the theory that differences in rate of repolarization between epicardial and endocardial myocardium are responsible for the formation of the T wave.

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EFFECTS OF PERCUTANEOUS STIMULATION ON THE CIRCULATION IN NORMAL AND IN PARALYZED LOWER EXTREMITIES

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IT IS generally recognized that voluntary muscular activity produces an increase of the circulation of the muscles. However, it is not clearly known what effect percutaneous electric stimulation would have on the blood flow in normal and in paralyzed (spastic and flaccid) human extremities. The purpose of this study was to investigate *a*) whether, and to what degree, percutaneous electric stimulation of normally innervated muscles would increase the circulation in the stimulated extremity; *b*) what effect a similar stimulation of extremities (spastic and flaccid) deprived of voluntary activity would have on their circulation.

In 1877 Gaskell (1) observed a great increase of venous outflow at the beginning of tetanic contraction of muscles. The outflow then diminished while the stimulus was continued. He reported that a greater amount of blood was supplied per unit of time to a rhythmically contracting muscle than to a resting one.

Benedict and Parmenter (2) obtained a reduction of cutaneous temperature of the extremities after a period of stair-climbing or walking on a treadmill in spite of the associated increase of production of heat as a result of the augmentation of metabolic rate. They explained these findings by stating that after exercise vasoconstriction of cutaneous vessels occurs and more blood from the periphery goes to the activated muscles. On the contrary, Grant and Pearson (3) obtained an increase of the temperature of the skin over contracting muscles during rhythmic exercise. This was considered as a transfer of some of the heat to the skin from the underlying muscle well heated from activation. Eggleton (4) stated that the temperature of the skin over an active muscle may rise more than 1° C., while Lewis and Pickering (5) noted that exercise of the hypothenar muscles led to warming of the ulnar side of the hand by 2° or 3° C. within ten minutes.

Grant (6) investigated plethysmographically the circulation in the forearm alone after producing arterial occlusion at the wrist. He found that immediately after contraction the blood flow increased and was sustained at a maximal level. Immediately after relaxation the flow increased enormously and then declined to control level. He reported an increase of the temperature of the skin over tonically contracted muscles.

Kramer and Quensel (7) obtained an increase of blood flow even during the time that the muscle remained contracted. This was confirmed by Bülbring and Burn (8).

By the use of the thermostromuhr on the femoral artery in anesthetized dogs, Rein and associates (9, 10) obtained a great increase of blood flow in the artery during the application of short rhythmic tetanic stimuli on the muscle. However, during prolonged tetanic stimulation the blood flow abruptly increased at first and gradually returned to the original level.

Received for publication February 16, 1948.

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By the use of the hot wire anemometer in anesthetized animals Anrep and associates (11) observed that during tetanic stimulation of the motor nerves there was a mechanical compression of the intramuscular vessels for a short period during which the arterial inflow into the muscles was greatly diminished or arrested. They stated that the blood flow was increased in muscles between contractions.

METHODS

The stimulator described by Paul and Couch (12) was used for percutaneous electric stimulation of the muscles in the lower extremities of normal and of paralyzed human subjects. The stimuli were produced by condenser discharges at a frequency of 90 cycles per second with an intensity varying with the tolerance of the subject.

The electrodes, one 4 by 5 inches (10 by 13 cm.) and the other 5 by 8 inches (13 by 20 cm.), were soaked in a solution made up of 3 per cent lactic acid and 17 per cent alcohol in water and were applied to specific areas of the extremity in which the blood flow was determined. The electrodes were applied, one to the dorsal aspect of the calf muscles, and the other just above the ankle. In a few cases the position of one electrode was alternated between the ventral and the dorsal aspect of the limb in order to determine whether such a change of position of the electrodes would produce any different effect.

The temperatures of the skin were recorded galvanometrically by thermocouples applied to the internal aspect of the calf of the leg before and after stimulation.

The blood flow in the extremities was determined by the use of venous occlusion plethysmographs with a compensating spirometer recorder (13). The subject was placed comfortably on the test bed and the plethysmographs were applied to each of the extremities including the foot and leg up to one inch (2.5 cm.) below the tibial tuberosity. After a period of about twenty minutes in which the subject became adjusted to the plethysmographs and the surroundings, control cutaneous temperatures and determinations of blood flow were obtained. The plethysmographs were removed and electric stimulation was given to one extremity for fifteen minutes while the contralateral unstimulated extremity was used as control. At the end of the period of stimulation the extremities were again introduced into the plethysmographs, and blood flow and cutaneous temperatures were determined in both the stimulated and the unstimulated extremity. The percentage change in blood flow was calculated in every case from the total blood flow values and not from the values calculated per 100 cc. of tissue.

RESULTS

This study was made on 22 volunteer human subjects: 8 normals, 7 with spastic and 7 with flaccid lower extremities. Eight observations were performed on normal extremities, 22 on spastic extremities and 16 on flaccid extremities, making a total of 46 observations.

The average control blood flow per 100 cc. of tissue in the lower extremities was 3.4 cc. for the spastic subjects, 3.2 cc. for the normals and 3.8 cc. for the flaccid subjects. Under the conditions of this study, the blood flow values in normal, spastic and flaccid lower extremities before stimulation did not differ significantly when considered per 100 cc. of limb volume.

After electric stimulation the spastic lower extremities showed an increase of blood flow in every observation (table 1). The average increase in flow over the control was +111 per cent with a range from +32 per cent to +340 per cent. The changes of cutaneous temperature were inconsistent; the temperatures were reduced in 3 of 21 observations and increased in the rest. After stimulation, the spastic patients experienced a reduction of spasticity, which varied in magnitude and duration.

TABLE 1. EFFECTS OF PERCUTANEOUS ELECTRIC STIMULATION ON BLOOD FLOW AND CUTANEOUS TEMPERATURE IN SPASTIC LOWER EXTREMITIES

OBSERVATION	STIMULATED EXTREMITY				UNSTIMULATED EXTREMITY			
	Blood flow, cc. per 100 cc. of tissue				Blood flow, cc. per 100 cc. of tissue			
	Before stimulation	After stimulation	Percentage change	Change in skin temperature	Before stimulation	After stimulation	Percentage change	Change in skin temperature
				°C.				°C.
21	2.0	3.4	+72	+1.1	2.3	2.3	0	-0.6
20	1.7	3.5	+108	+1.0	1.5	2.1	+43	-0.5
30	4.3	5.6	+32	0	3.0	2.8	-5	+0.5
3	4.8	7.9	+65	+1.5	2.9	4.0	+40	+1.3
38	2.6	4.6	+76	+1.3	1.7	1.7	-2	-1.9
28	1.9	4.4	+133	-1.4	2.7	1.9	-30	-0.6
54	4.3	5.9	+36	+1.7	3.7	3.7	+2	+0.9
13	1.7	2.7	+58	+1.8	2.4	2.9	+21	+1.1
6	4.3	7.1	+64	+0.3	3.8	2.8	-25	+0.7
19	3.7	6.3	+73	+2.9	3.3	2.8	-15	+0.1
26	4.2	9.6	+129	+1.5	3.7	2.2	-42	+0.6
18	2.6	6.0	+133	+0.6	2.0	1.2	-42	-0.1
1	3.6	10.4	+188	+3.2	1.9			+2.0
10	2.2	5.3	+143	+1.5	2.7	3.1	+17	+0.5
2	2.3	7.7	+231	+1.3	3.1	1.7	-46	-0.1
53	5.1	13.0	+155	-0.2	1.9	3.2	+65	+0.3
58	2.6	11.2	+340	+0.8	5.2	10.0	+92	+0.2
59	2.2	6.2	+176	+0.9	7.7	6.2	-19	+0.2
4	5.0	7.2	+44		4.3	4.6	+6	
50	4.1	6.1	+49	-0.2	4.7	5.6	+18	+0.7
8	4.6	6.8	+50	+1.1	5.3	4.3	-19	-0.1
11	4.5	8.1	+79	+0.2	4.3	6.3	+46	-0.2
Average	3.4	6.8	+111	+0.9	3.5	3.6	+5	+0.2

The change of blood flow and of cutaneous temperature in the unstimulated spastic extremity were inconsistent (table 1). The spastic subjects tolerated a stimulus of considerably higher intensity than that applied to the normals.

In the spastic subjects, the extremity to which the electrodes were applied responded vigorously during the period of stimulation, but the contralateral limb was not always absolutely quiet. It gave a number of spontaneous movements. The increase of blood flow that occurred in a few of the unstimulated spastic extremities is attributable to such active spontaneous movements. Figure 1 demonstrates blood

flow curves in the treated and the untreated extremity before and after electric stimulation.

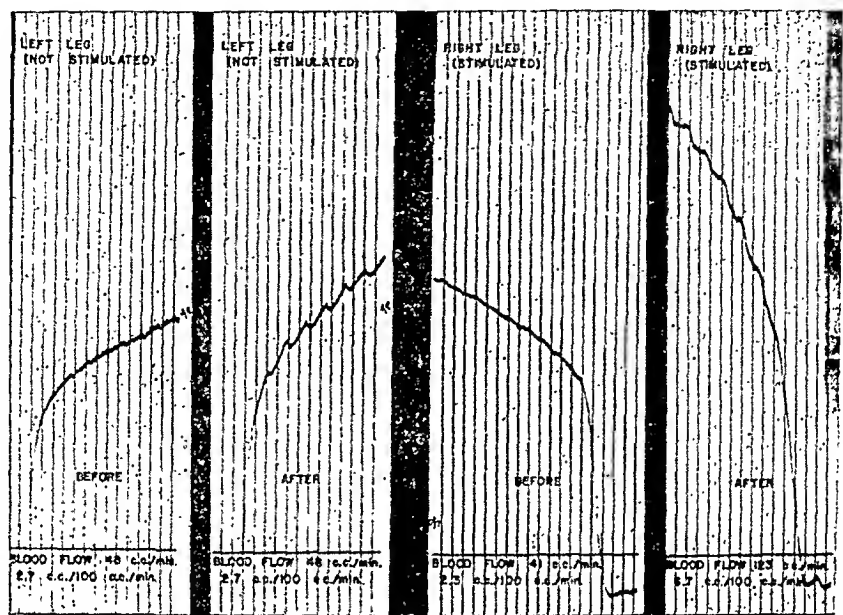


Fig. 1. EFFECT OF PERCUTANEOUS STIMULATION ON BLOOD FLOW. Blood flow curves before and after electric stimulation of spastic muscles. The flow curves of the unstimulated contralateral extremity are included for comparison.

TABLE 2. EFFECTS OF PERCUTANEOUS ELECTRIC STIMULATION ON BLOOD FLOW AND CUTANEOUS TEMPERATURE IN NORMAL LOWER EXTREMITIES

OBSERVATION	STIMULATED EXTREMITY				UNSTIMULATED EXTREMITY			
	Blood flow, cc. per 100 cc. of tissue				Blood flow, cc. per 100 cc. of tissue			
	Before stimulation	After stimulation	Percentage change	Change in skin temperature °C.	Before stimulation	After stimulation	Percentage change	Change in skin temperature °C.
42	2.8	3.0	+10	-0.1	3.1	2.8	-10	0
24	3.6	4.6	+28	-0.2	3.5	1.8	-47	-0.5
5	6.4	9.5	+49	0	4.0	5.5	+36	-0.4
22	2.1	3.1	+49	-0.1	2.3	1.8	-22	-1.2
63	2.3	4.4	+95	+0.4	1.5	1.4	-6	-1.0
23	4.2	8.9	+115	-0.4	6.0	7.7	+28	-0.7
25	2.8	6.7	+136	-0.4	3.4	4.2	+25	0
61	1.9	6.0	+210	-0.4	2.1	1.7	-17	-1.0
Average	3.2	5.8	+86	-0.1	3.2	3.3	-2	-0.6

The blood flow in normal lower extremities of healthy subjects consistently increased as a result of electric stimulation (table 2). The average increase over the control flow was +86 per cent, with a range from +10 per cent to +210 per cent.

Figure 2 presents blood flow curves in the normal treated and untreated extremities before and after stimulation. The increase of blood flow in the normal extremities

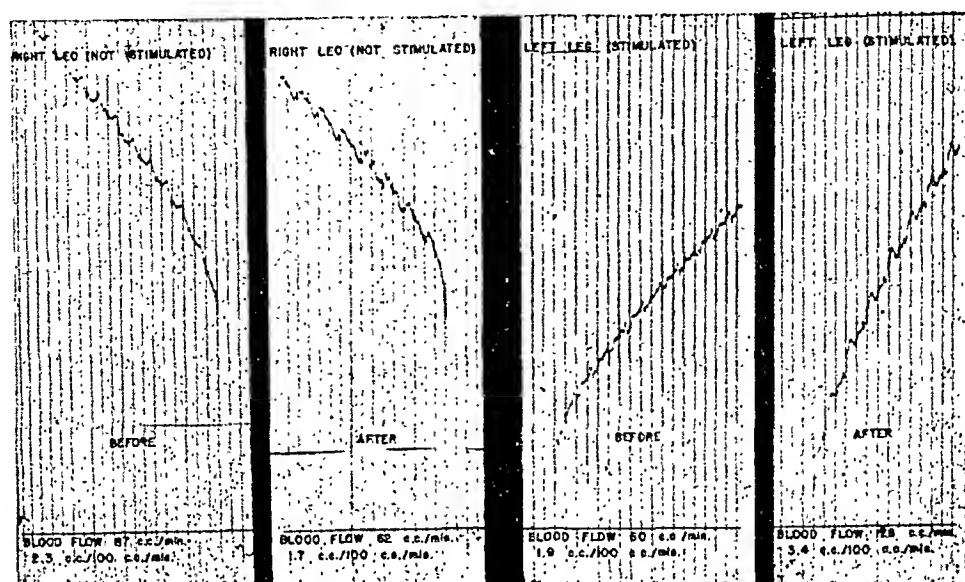


Fig. 2. BLOOD FLOW CURVES BEFORE AND AFTER ELECTRIC STIMULATION OF NORMAL MUSCLES. The flow curves of the unstimulated contralateral extremity are included for comparison.

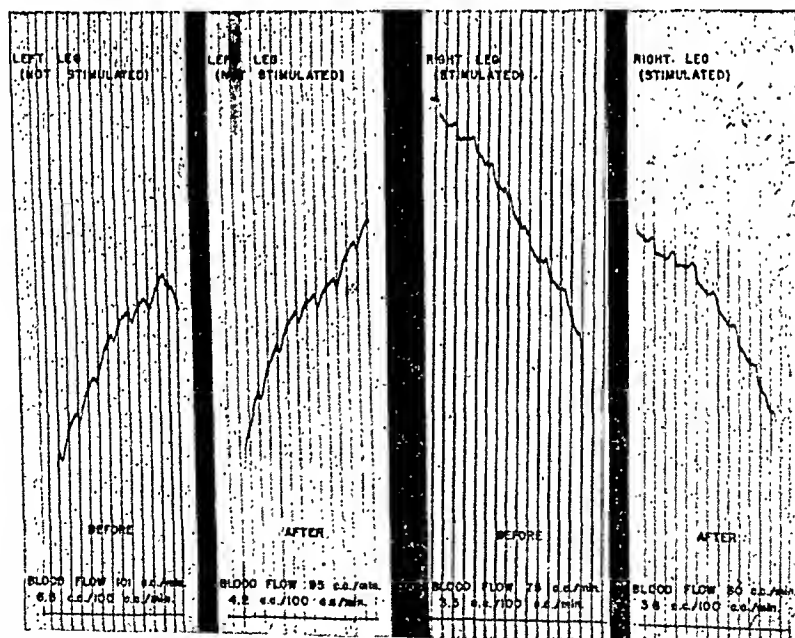


Fig. 3. BLOOD FLOW CURVES BEFORE AND AFTER ELECTRIC STIMULATION OF FLACCID MUSCLES. The flow curves of the unstimulated contralateral extremity are included for comparison.

is less than that in the spastic extremities as a result of electric stimulation. This is attributable to the fact that normal subjects could not tolerate stimulation with as great an intensity as that applied to the spastic subjects. When the intensity of the

current was increased beyond a certain range, painful contractures occurred in the normal extremity. Of the eight normal subjects, six showed after stimulation a reduction of cutaneous temperature of the stimulated extremity, one showed no change and one showed an increase. The changes of blood flow in unstimulated lower extremities were inconsistent and the average change was insignificant, but the cutaneous temperatures were unchanged in two subjects and significantly reduced in the rest.

Sixteen observations were performed on the flaccid lower extremities of seven subjects who had lower motor neuron paralysis. None gave a vigorous response,

TABLE 3. EFFECTS OF PERCUTANEOUS ELECTRIC STIMULATION ON BLOOD FLOW AND CUTANEOUS TEMPERATURE ON FLACCID LOWER EXTREMITIES

OBSERVATION	STIMULATED EXTREMITY				UNSTIMULATED EXTREMITY			
	Blood flow, cc. per 100 cc. of tissue				Blood flow, cc. per 100 cc. of tissue			
	Before stimulation	After stimulation	Percentage change	Change in skin temperature	Before stimulation	After stimulation	Percentage change	Change in skin temperature
				°C.				°C.
44	2.1	3.1	+48	+1.6	3.3	3.6	-10	-1.2
32	8.5	7.9	-7	-1.0	8.6	6.6	-23	+1.0
29	3.4	3.5	+3	+1.5	2.2	3.1	+44	+1.0
31	5.9	8.3	+42	-0.9	6.8	4.5	-33	+0.7
35	7.1	13.0	+83	+0.1	6.2	8.5	+37	+0.6
14	5.5	3.2	-41	+1.7	3.7			+0.1
7	2.6	2.6	0	-0.7	2.6	3.3	+28	+0.2
66	2.8	3.5	+26	-1.4				-0.7
56	2.4	3.2	+32	-1.0	2.7	3.8	+42	0
3	2.7	2.0	-26	+0.8	4.3	3.7	-14	+0.5
48	2.4	3.2	+33	+1.3	2.1	3.0	+44	+0.6
34	3.4	3.7	+9	-1.2	3.0	3.2	+8	+0.1
33	4.1	4.9	+21	-0.9	4.4	5.0	+13	0
27	1.8	2.6	+43	-0.4	2.3	3.1	+33	-0.2
36	2.0	2.8	+37		2.3	3.5	+52	
40	4.3	3.1	-28	-1.6	3.8	3.8	0	+0.2
Average	3.8	4.4	+17	-0.1	3.9	4.2	+16	+0.2

a few showed perceptible contractions and no reactions could be observed in the rest. After stimulation, four subjects showed a reduction of blood flow, one showed no change and the rest showed a slight increase; the average was +17 per cent and the range was from -41 per cent to +83 per cent. Figure 3 shows blood flow curves of the treated and untreated flaccid extremities before and after stimulation. Before and after electric stimulation the cutaneous temperatures were recorded in 15 observations. In nine they were decreased after stimulation, and in six increased.

Comparison of the data (table 3) obtained on blood flow in the stimulated and unstimulated flaccid extremities indicates that the changes are of practically the same magnitude, thus signifying the ineffectiveness of electric stimulation of flaccid muscles on blood flow in that extremity.

SUMMARY

By means of the venous occlusion plethysmograph with a compensating spirometer recorder the effects of electric stimulation on blood flow in the normal and paralyzed lower extremities of 22 human subjects were studied. Cutaneous temperatures over the ventral aspect of the calf region of the legs were recorded before and after stimulation.

All the observations on the spastic lower extremities gave as a result of stimulation a definite increase of blood flow, which averaged 111 per cent and ranged from 32 per cent to 340 per cent more than the control value. After stimulation the spastic subjects experienced a reduction of their spasticity, which varied in magnitude and duration.

Similarly, after stimulation the blood flow in the treated extremities of normal subjects showed an increase, which averaged 86 per cent more than the control and ranged from 10 per cent to 210 per cent. The smaller magnitude of increase of blood flow in the extremities of normal subjects is attributed to their inability to tolerate as strong a stimulus as the spastic ones.

Electric stimulation of flaccid muscles produced insignificant changes of blood flow.

The increase of blood flow in the stimulated extremities, the muscles of which contracted vigorously on electric stimulation, and the insignificant increase or no change of blood flow in extremities, the muscles of which gave weak contractions or none on stimulation, strongly indicate that the increase of blood flow is chiefly contributed through the activated muscles.

We gratefully acknowledge the excellent technical assistance of Mr. Adrien N. Porter.

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INFLUENCE OF DIURESIS ON UREA PRODUCTION IN THE FASTING DOG¹

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THE production of exogenous urea varies with the protein intake; major catabolic stimuli such as exercise, starvation, fever and injury are known to increase endogenous urea production. This common knowledge rests on experiments in which urea elimination into the bladder was properly accepted as an adequate index of urea production. More rapid changes in urea production, however, have received little attention and it has been tacitly believed that the fasting animal produces a relatively constant amount of urea except as the exhaustion of its glycogen and fat reserves leads to the greater use of body protein as a source of energy.

The experiments here reported suggest that urea metabolism is subject to rapid changes whose immediate cause and control is unknown but which may be studied by modifications of the method here used.

The general acceptance (1) of Marshall's finding (2) that urea is nearly uniformly distributed through the body water makes possible the use of changes in the urea content of plasma samples as an index of change in the total urea content of the body. If the changes in urea content and the urea output into the bladder are known, the production may be computed (production = output \pm change of urea content in total body water). These experiments examine the constancy of urea production in dogs fasting with and without induced water diuresis.

Ogden (3) called attention to the fundamental relationship between urea output, urea production, total body urea and total body water by deducing the following formula:

$$W = \frac{(UV)_2 - (UV)_1}{P_1 - P_2} \cdot \frac{T}{10}$$

where:

- W = total body water (liters),
- P₁ = mgm. urea in 100 cc. plasma water (start),
- P₂ = mgm. urea in 100 cc. plasma water (end),
- (UV)₁ = urea output before diuresis (mgm./min.),
- (UV)₂ = urea output during whole diuresis (mgm./min.),
- T = total duration of diuresis (minutes).

This formula assumes the constancy of the urea production and the uniformity of urea distribution in the body water. If the total water (W) be assumed as 70 per cent of the body weight, the same relationship can be used to test the constancy of urea production and to evaluate its changes quantitatively.

METHOD

Trained unanesthetized dogs (female) fasted for 24 hours were catheterized. Urine was collected continuously with no interval between the periods during which

Received for publication March 2, 1948.

¹ Grateful acknowledgment is made for assistance from the John and Mary R. Markle Foundation.

urine flow was measured and sampled. At the end of each period the bladder was rinsed with several washes of water, this water being added to the collected urine. When the collection periods exceeded three hours the animal was released from the dog board and placed in a metabolism cage. At the end of the period the dog was recatheterized and the bladder washings added to the collected urine. While on the dog board these animals were given continuous attention to keep them relaxed.

Blood samples (3 cc.) were drawn from the jugular vein and immediately analyzed for urea by the method of Van Slyke (4).

All the blood urea concentration figures here reported are in terms of mgm. urea/100 cc. blood water. Approximately 250 mgm. of blood from each sample were absorbed on weighed filter paper, weighed and dried to constant weight in a vacuum desiccator.

Urine samples were analyzed for urea according to the method of Van Slyke (4).

RESULTS

Rate of urea production during fasting. Figure 1 (B, C and D) presents the blood urea concentration (expressed as mgm. urea/100 cc. blood water) and the rate of urea excretion (UV) in three fasting dogs.

In dog 1 (B) during the first three hours (181 min.) of the experiment the urea concentration in the plasma water was unchanged (41.5 mgm. %); therefore, the total urea production during this time was equal to the total urea recovered from the bladder (609 mgm.) and the average rate of urea production equal to 609/181 or 3.36 mgm./min. (UV). This reasoning assumes uniform distribution of urea in the body water at the moments of the two blood samples.

During the next nine and a half hours (571 min.) the urea concentration in the blood water declined from 41.5 to 37.4 mgm/100 cc., suggesting that the production of urea was not keeping pace with the elimination. The elimination during this time totaled 1.672 grams. Therefore, the urea production was probably less than 1.672 grams during these nine and one half hours or less than 2.93 mgm/min. This is definitely less than the previous rate of urea production (3.36 mgm/min.).

If we assume that water was 70 per cent (5) of the body weight (12.5 kgm.), this animal had 8.75 kgm. water diluting the urea. The fall in blood urea from 41.5 to 37.4 mgm. per cent would have entailed an excretion of 358 mgm. urea in excess of the production which must therefore have approximated 1.672 grams—1.314 grams in nine and one half hours or 2.30 mgm/min. This is a reasonable agreement with the conclusion (urea production < 2.93 mgm/min.) of the previous paragraph.

During the subsequent nine hours (532 min.) the rate of urea elimination was about the same as before (UV = 2.78 mgm/min.). But during this time the urea concentration in the blood water rose from 37.4 to 41.1 mgm. per cent. This (assuming 70 per cent water) indicates a retention of 322 mgm. urea, so that production during this period must have been 1470 mgm. + 322 mgm. or 1.792 grams in nine hours or 3.36 mgm./min.

Table 1 presents the calculations made according to the above reasoning with respect to each urine collection and blood sample taken from these three dogs. For simplicity of reasoning in the foregoing paragraphs the whole of the experiment

on *dog 1* was recalculated on the basis of three long subdivisions. This accounts for the minor differences between the figures above and the figures in the table. No attempt was made in the calculations to take into account the change in total body

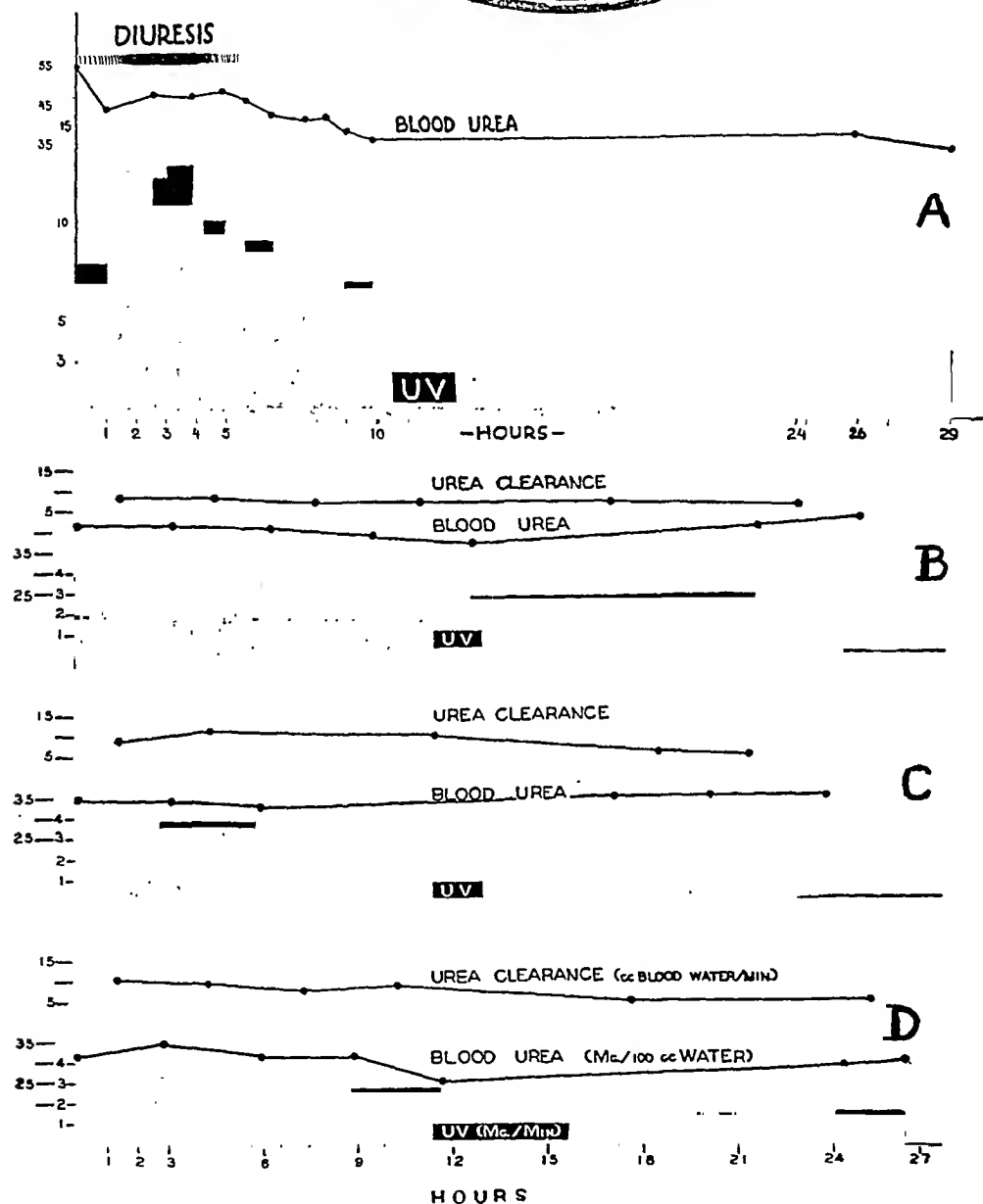


Fig. 1. INFLUENCE OF DIURESIS AND FASTING UPON THE BLOOD UREA AND UREA ELIMINATION. (A) *dog 1*, diuresis experiment (water given at 0 time); (B), (C), and (D) *dogs 1, 2, and 3*, fasting without water.

water due to known losses through the bladder and unknown losses through the lungs during the experiment.

The figures in table 1 for computed urea production (column 9) indicate that there are large changes of the order of 50 per cent or more of the initial values in the computed urea production rate. Those periods in which the blood urea level

(column 5) changed very little (*dog 1*, period 1; *dog 2*, period 4; *dog 3*, period 2) may be taken as having greater precision since they depend in essence only upon the measurement of a large amount of urea collected from the bladder. In proportion as the correction (column 6) for blood urea changes becomes greater, greater inaccuracies are liable to creep into computation of urea production. In spite, however, of the complication of this method of determining the rate of urea production and the possibility of error in the data from which the computations are made, the changes

TABLE 1. UREA PRODUCTION IN FASTING DOGS WITHOUT WATER

DOG 1	PERIOD 2	TIME 3	V 4	BW _b 5	TOTAL CHANGE 6	E 7	TOTAL PRODUCTION 8	RATE OF PRODUC- TION 9	UV 10
		min.	cc/min.	mgm/100 cc. water	mgm.				mgm/ min.
1 (12.5 kgm)	1	181	0.0468	41.5	+8.75	609	617.75	3.42	3.38
	2 ¹	179	0.0476	41.6	-61.2	584	522.8	2.92	3.27
	3	204	0.064	40.9	-175.0	576	401.0	1.97	2.82
	4	189	0.068	38.9	-131.0	512	381.0	1.98	2.70
	5	533	0.066	37.4	+324.0	1470	1794.0	3.37	2.80
	6	190	0.046	41.1 42.6 ²	+131.0	465	596.0	3.14	2.46
2 (11.0 kgm)	1	166	0.0574	34.9	-23.1	534	510.9	3.08	3.21
	2 ¹	181	0.0610	34.6	-84.6	705	620.4	3.42	3.90
	3	673	0.0475	33.5	+131.0	2430	2561.0	3.81	3.62
	4	169	0.0530	35.2	0	370	370.0	2.19	2.18
	5	180	0.0528	35.2 34.8 ²	-30.8	314	283.2	1.57	1.75
3 (6.5 kgm)	1	346	0.0692	31.6	+27.3	1183	1210.3	3.50	3.39
	2 ¹	184	0.055	32.2	-4.6	477	472.4	2.57	2.58
	3	167	0.060	32.1	-182.0	451	269.0	1.61	2.70
	4	749	0.035	28.1	+59.1	1060	1119.1	1.50	1.42
	5	144	0.035	29.4 30.7 ²	+59.1	232	291.1	2.02	1.62

¹ Period during which 12:00 noon occurred.

² Sample at end of experiment.

Time—duration of urine collection; V—urine flow; BW_b—urea in blood water at beginning of period; Total change—change in blood urea concentration \times 70% body weight; E—total urea eliminated in bladder (UV \times T); Total production—elimination \pm total change; Rate of production—total production/time; UV—Rate of urea elimination into bladder.

are so great that they cannot be ignored. Moreover, their variations do not appear to be random.

This experiment demonstrates that urea metabolism is subject to variable changes in response to unrecognized circumstances arising during a 23- to 27-hour period of observation. There is some suggestion in these figures that the urea production is higher at night than in the day-time. Moreover, there is a tendency for the plasma urea concentration to fall during the day and to be restored during the night. This suggests that the changes in urea production are not caused by the dehydration resulting from withdrawal of water since changes from such a cause would likely be progressive.

TABLE 2. UREA PRODUCTION IN FASTING DOGS AFTER WATER

DOG 1	PERIOD 2	TIME 3	V 4	BW _b water 5.	TOTAL CHANGE 6	E 7	TOTAL PRODUCTION 8	RATE OF PRODUC- TION 9	UV 10
		min.	cc./min.	mgm./100 cc.	mgm.				mgm./ min.
Water									
1	1	78	1.25	54.9	-960.0	1203	243	3.12	7.59
	2	79	4.88	43.9	+288.0	1020	1308	16.6	12.25
	3	73	4.48	47.2	-35.0	920	885	12.1	12.71
	4 ¹	69	3.06	46.8	+78.5	667	746	10.8	9.66
	5	37	0.603	47.7	-157.0	311	154	4.2	8.50
	6	125	0.256	45.9	-394.0	1011	617	4.9	8.10
	7	77	0.130	41.4	-306.0	508	202	2.6	6.60
	8	36	0.222	37.9	-201.0	248	47	1.3	6.87
	9	960	0.15	35.6	+43.6	3780	3824	3.9	3.94
	10	200	0.17	36.1	-288.0	684	396	1.98	3.42
				32.8 ²					
2	1	77	0.95	30.3	+7.7	299	307	3.99	3.90
	2	36	0.96	30.4	-69.2	129	60	1.67	3.54
	3	42	0.119	29.5	-115.0	166	51	1.21	3.96
	4 ¹	30	0.145	28.0	-15.4	125	110	3.66	4.20
Water									
	5	54	1.32	27.8	-354.0	452	98	1.82	8.37
	6	44	5.04	23.2	-123.0	453	330	7.5	10.3
	7	44	4.38	21.6	-154.0	268	114	2.59	6.1
	8	45	3.20	19.6	-69.2	222	153	3.40	4.96
	9	63	0.716	18.7	-162.0	234	72	1.14	3.69
	10	29	0.342	16.6	0	112	112	3.86	3.84
	11	56	0.17	16.6	-85.0	159	74	1.32	2.84
	12	21	0.16	15.5	+69.2	54	123	6.22	2.63
				16.4 ²					
3	1	64	0.133	24.7	+86.5	191	278	4.34	2.97
	2	63	0.151	26.6	+91.2	190	281	4.47	3.02
	3	63	0.103	28.6	-22.8	151	128	2.04	2.40
Water									
	4 ¹	37	0.433	28.1	-177.0	216	39	1.06	5.85
	5	34	4.37	24.2	-91.2	343	252	7.41	10.00
	6	49	3.83	22.2	-13.6	115	101	2.02	2.34
	7	66	1.80	21.9	-50.2	189	139	2.11	2.86
	8	44	1.15	20.8	-86.5	248	161	3.67	5.72
	9	92	0.36	18.9	-13.6	240	226	2.46	2.40
	10	84	0.137	18.6	-105.0	136	31	.37	1.62
				16.3 ²					

See legend to table 1.

Influence of water diuresis on urea production. In seven experiments on the same three dogs, 100 ml. water per kgm. of body weight were given by stomach tube (fig. 1, A). The experiments were then continued essentially in the same fashion as those in which no water had been given. The rate of urea production was computed

as before. No account was taken of the changing water content of the body as the result of the gavage or the loss of water due to the diuresis.

The comparison of the rate of urea production in these same dogs (table 2) after water as contrasted with the control experiments shows that the computed rates are much more variable. In every case (including the experiments omitted from the table) a high rate of urea production was found early in the diuresis at a time when the rate of urea elimination from washing out of urea was at its greatest. This was commonly followed by a period in which the urea production was depressed.

In those experiments where the periods of observation were short, it was common to find periods of apparently high urea production alternating with periods in which the computation gave negative values. Since the acceptance of these negative values leads to the unlikely conclusion that urea is being intermittently destroyed, the rapid apparent variations were interpreted to indicate that during the rapidly changing events in these experiments equilibrium between the urea content of the plasma and that of the tissues was unsteady. The premise that a plasma sample is representative of the tissues with respect to the urea concentration within its water is sound only when no rapid changes are taking place.

After the diuresis was complete the blood urea concentration usually continued to diminish even though the rate of urea production was low. At this time the blood urea level and the low rate of urine flow combined to reduce the rate of urea elimination as might be expected. Nevertheless, even the low rate of elimination was still apparently greater than the catabolic production of urea. In the one experiment in which the phenomena were followed for many hours after the completion of the diuresis (table 2, no. 1) it seemed that the rate of urea production was returning toward what may be regarded as normal for that animal.

Saline and glucose diuresis. A series of experiments was performed in which enormous diuresis was produced by the intravenous injection of hypertonic glucose and saline, dehydration being combated by water gavage.

In these experiments also it was evident that changes in the rate of urea production occurred. Here, too, it was clear that changes in blood urea content and in urea elimination persisted after the conclusion of the diuresis.

CONCLUSION

The rate of urea production in the fasting animal is relatively steady; such variations as occur, however, do not appear to be due to chance errors of measurement. In the animal under the influence of water diuresis, by contrast, the fluctuations are great and rapid. The disturbance continues long after the diuresis is complete. The first effect of the water diuresis seems to be to increase the rate of urea production as well as its rate of elimination from the body. Later the rate of production is much depressed.

DISCUSSION

The changes in blood urea content and urine urea output after water administration cannot be wholly explained by a simple theory of diuresis, 'washing out' the urea from the blood. Addis (6) suggested a similar conclusion on the basis of the

time lag between diuresis and maximum urea elimination in his experiments. These experiments also raise the question whether during the water diuresis and for a while after it, the rates of change are such that there is not equilibrium between the various tissues with respect to the urea concentration in their water.

In spite of the difficulties which this method presents because of the uncertainty that there is always urea equilibrium throughout the body water, the method is capable of indicating quantitatively changes in urea production occurring in an hour or less in response to diuresis and therefore presumably in response to other stimuli. The timing of these experiments was adjusted only to two purposes, the control of the magnitude of chance diurnal variations and the study of a single short large diuresis. For the elucidation of the exact mechanism by which the rate of urea production is controlled it would be desirable to produce less violent displacements and to examine their effects by shorter test periods.

It is possible that the variations we observed in the experiments without diuresis may have been connected with the change from restraint supine on a dog board to the moderate freedom of a metabolism cage. The changes in the diuretic experiments might be due to adrenocortical disturbance activated by the violent changes in water and mineral metabolism. These possibilities remain to be investigated.

Upon the two concepts, the uniformity of distribution of urea and the constancy of its rate of production, depend the determinations of total body water by the method of Painter (7) and the computation of body water by dilution of urea according to the formula proposed by Ogden. Any body water figures depending on urea distribution must be interpreted with these two limitations in mind.

SUMMARY

Blood urea level and total urea elimination into the bladder were measured in unanesthetized dogs. Each experiment, lasting 6 to 29 hours, was subdivided into urine collection periods of appropriate duration. The dogs were subjected on different days to: 1) fasting and deprivation of water; 2) fasting and a large gavage of water; and 3) fasting, water and intravenous injection of hypertonic saline and glucose.

From these data and the assumption that 70 per cent of the body consists of water, the rate of urea production (as distinct from urea elimination) was calculated. This was found to be slightly variable in the animals without water and showed a marked rise occurring with the diuresis in the animals given water. This rise in urea production was distinct from the washing out of urea which tended to occur with diuresis.

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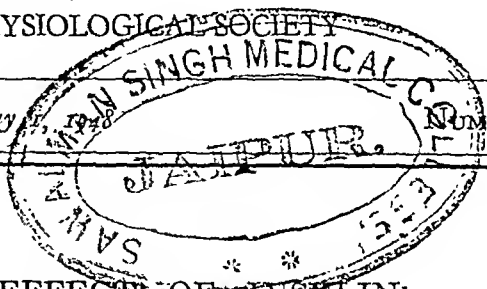
THE AMERICAN JOURNAL OF PHYSIOLOGY

Published by
THE AMERICAN PHYSIOLOGICAL SOCIETY

VOLUME 153

May 1, 1948

NUMBER 2



HYPERGLYCEMIC EFFECT OF INSULIN¹

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THE existence of substances of pancreatic origin which are capable of producing hyperglycemia has been recognized for many years. In 1923, Murlin, Clough, Gibbs and Stokes (1) found that a hyperglycemic material could be obtained from pancreas, and in the same year Fisher (2) demonstrated a blood sugar-elevating factor in insulin preparations produced by the Somogyi method. In 1927 Martino (3) described the preparation from pancreas of a hyperglycemic principle which he later (4) showed to be identical with the substance which Bürger obtained from commercial insulins. About the same time, Funk (5) reported the dissolution of insulin into hyperglycemic and hypoglycemic fractions, although his method was not described. Between 1928 and 1937 Bürger and his co-workers published extensive observations on the hyperglycemic activity of insulin preparations (6-8). It is recognized that, following the intravenous administration of insulin, a hyperglycemic phase of variable duration precedes the fall in blood sugar.

Bürger, having shown that this phenomenon was less evident in crystalline than in amorphous preparations, concluded that it must represent the action of a separate substance present only in the amorphous material. He demonstrated further that whereas boiling with dilute alkali completely destroyed the hypoglycemic activity of insulin, the hyperglycemic effect was undiminished by such a procedure (7). Bürger's substance, which he called 'glukagon', was considered a separate and necessary internal secretion. He demonstrated that its activity was not mediated by the adrenal medulla, but concluded on the basis of injections into the portal vein that it acted directly on the hepatic glycogen reserves (6). He believed glukagon to be protein, very similar in chemical properties to insulin. A recent report of Heard *et al.* (9) mentions a hyperglycemic, glycogenolytic material which can be separated from crude insulin preparations by boiling with alkali.

Received for publication April 14, 1948.

¹ The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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There exists, of course, no direct proof that the hyperglycemic substances which have been thus far obtained are endocrine products normally produced by the pancreas. There are however certain physiological data which strongly suggest that the pancreas may elaborate a metabolic hormone distinct from insulin and that part of its function may include insulin antagonism. Young (10), for example, found that dogs with permanent diabetes following a course of anterior pituitary injections exhibited very high insulin requirements although they could survive for remarkably long periods when insulin was not given. Similarly Thorogood and Zimmermann (11) found that alloxan diabetic dogs required extremely large dosages of insulin for prevention of glycosuria whereas after the same animals were subjected to pancreatectomy their insulin requirements were markedly reduced. As with Young's dogs, however, the alloxan animals could survive for long periods without insulin and did not develop ketosis. Following pancreatectomy they developed ketosis and coma when insulin was withheld. Since, in both of these preparations, the beta cells are found to be largely destroyed with little or no damage to the alpha cells, the latter have been suggested as the source of a principle which antagonizes insulin's action and at the same time counteracts ketogenesis. Some such mechanism could also explain the large insulin dosage required by many diabetics and the relatively low insulin requirements which have been described in totally pancreatectomized human beings (12).

It seems important, therefore, to explore various types of pancreatic extracts with regard to hyperglycemic activity, effect on ketone body formation, and insulin antagonism. Our attention was directed primarily to the hyperglycemic factor of commercial insulin.

MATERIALS AND METHODS

For amorphous insulin, U 40 'regular' commercial insulin was used.⁴ The crystalline preparation was dry zinc-insulin crystals without preservative.⁵ Inactivation was carried out by reduction of the disulfide linkages with cysteine, a reaction which was described by Du Vigneaud (13) and has been studied in detail by Wintersteiner (14). Two mgm. of cysteine were used for each milligram of insulin in 0.2 per cent solution. The reaction was carried out at pH 8.5 in an atmosphere of nitrogen at room temperature for 24 hours. The protein was then precipitated by saturation with ammonium sulfate, redissolved at pH 8.0 and dialyzed against water. Following this it was either lyophilized or dissolved by dialysis in isotonic saline at pH 2.5 in which it was administered.

Dogs were used which had been trained to tolerate repeated venepunctures without excitement. Three to five 'base line' samples were drawn before the material was injected and, in so far as possible, the same needle was left in place, its patency being maintained by the injection of very small volumes of saline.

Blood glucose determinations were carried out by the micro method of Folin (15) and blood acetone body levels were measured by a modification of the Behre method which we have described (16). This method measures the total of acetone and acetoacetate in terms of acetone.

Assays of insulin potency and of the residual activity of inactivated preparations were carried out by the mouse-convulsion method (17).

RESULTS

Hyperglycemic activity of crystalline insulin and inactivated amorphous and crystalline preparations in normal dogs. Although previous work had indicated that

⁴ ILETIN, manufactured by Eli Lilly and Company.

⁵ Lot No. 987267 prepared by Eli Lilly and Company.

crystalline insulin possessed no hyperglycemic activity (18), Olsen and Klein reported initial hyperglycemia with zinc-insulin crystals (19). In view of this, it seemed important to test the material which was available with reference to this activity. Table 1 shows the maximum hyperglycemia and its duration following intravenous administration of 0.1 to 2.0 units per kilo body weight. Initial hyperglycemia is apparently always present, but varies in degree and is of very short duration. There appears to be very little relationship between dosage and degree of hyperglycemia in this dosage range.

In 18 samples of amorphous and crystalline insulin inactivated by the method described, residual hypoglycemic activity as determined by mouse tests was only 0.1 per cent to 1.0 per cent of original. Table 2 shows the results of intravenous injection

TABLE 1. EFFECTS OF CRYSTALLINE INSULIN (LOT NO. 987267) ON BLOOD SUGAR IN NORMAL DOGS

DOSE	DURATION OF HYPERGLYCEMIA	MAXIMUM HYPERGLYCEMIA	MAXIMUM ELEVATION	MINIMUM BLOOD SUGAR
<i>mgm. per kilo</i>	<i>min.</i>	<i>% of initial</i>	<i>mgm. %</i>	<i>% of initial</i>
0.1	2	110	5.5	44.5
0.2	4	115	11.5	44.0
0.5	5	110	6.2	42.0
0.7	7	108	5.8	40.5
1.0	7	113	9.7	47.5
1.2	6	121	12.5	34.0
1.4	11	129	21.7	44.5
1.6	8	114	18.0	39.0
1.8	7	110	6.2	31.0
2.0	4	134	23.5	31.0

TABLE 2. COMPARISON OF EFFECTS OF INACTIVATED AMORPHOUS AND CRYSTALLINE INSULIN ON BLOOD SUGAR OF NORMAL DOGS

MATERIAL	NO. OF EXPER.	MAXIMUM HYPERGLYCEMIA		DURATION HYPERGLYCEMIA		MINIMUM BLOOD SUGAR	
		Mean	σ	Mean	σ	Mean	σ
		% of initial		minutes		% of initial	
Amorphous inactivated.....	10	144	10.4	24.2	11.1	92.6	7.92
Crystalline inactivated.....	10	137	16.1	21.4	7.09	89.3	5.16

tion of cysteine inactivated preparations. Figures 1 and 2 show sample curves from these groups. Although the mean maximum elevation in the amorphous is 44 per cent and, in the crystalline group 137 per cent, this difference according to Student's 'T' test, is not significant.

Blood sugar and ketosis in pancreatectomized dogs. Insulin administration was stopped 24 hours before an experiment, and no food was given for 12 to 18 hours. The results of experiments in which 0.1 milligram per kilo of inactivated amorphous insulin was given intravenously are summarized in table 3 and illustrated in figure 3. The hyperglycemia is not marked in pancreatectomized animals. The ketone levels tend to be elevated, however, during the hyperglycemic phase and follow the blood sugar closely.

Similar results were obtained with intact amorphous insulin given to diabetic dogs (table 3, fig. 4). The fall in both sugar and ketones was naturally accentuated but the time relationships were about the same.

Effect of inactivated insulin on insulin-induced convulsions in mice. In these experiments, varying amounts of inactivated insulin were injected subcutaneously into mice simultaneously with insulin. The mice were kept in cans in a constant temperature bath at 37°C. The results are illustrated in figure 5. The control (dotted) line represents the relationship between dosage of insulin and percentage convulsions. The other curves representing this relationship in the 'treated' groups do not deviate significantly from the control. Each point on the control curve represents 36 mice.

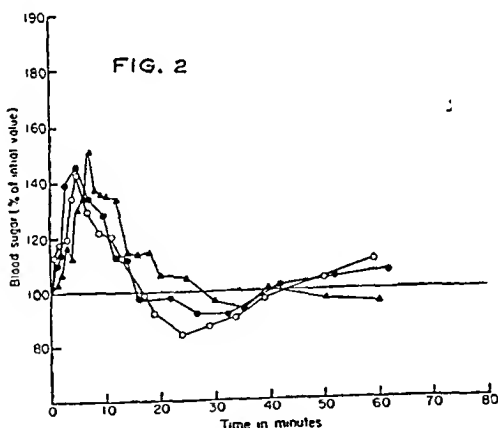
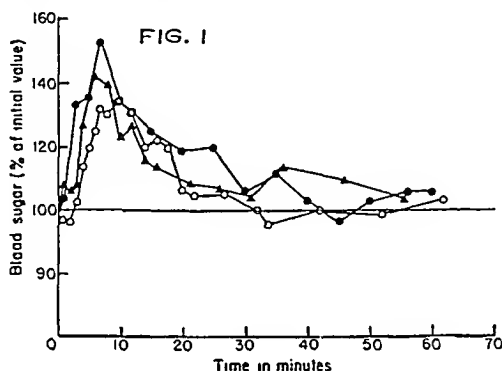


Fig. 1. THREE SAMPLE CURVES showing course of blood sugar in normal dogs following intravenous injection of 0.1 mgm/kilo of cysteine-inactivated amorphous insulin.

Fig. 2. THREE SAMPLE CURVES showing course of blood sugar in normal dogs following intravenous injection of 0.1 mgm/kilo of cysteine-inactivated crystalline insulin.

Each experimental point represents 24 to 28 mice. It appears then that the inactive material has no protective effect against insulin-induced convulsions.

DISCUSSION

From the similarity in results obtained by the inactivation of crystalline and amorphous insulin, it is apparent that the hyperglycemic activity of insulin is not, as was supposed by Bürger and others, the result of a contaminant peculiar to amorphous preparations. From these data alone, the question might arise as to whether the hyperglycemic effect is actually the action of a separate substance or whether it represents one of the normal functions of the insulin molecule itself. Evidence in

favor of the former mechanism exists in much of the work which has recently been carried out on 'Novo' insulin, a Danish preparation possessing no initial hyperglycemia at all.

Blood ketone levels rose in pancreatectomized animals for a few minutes following the injection of intact and inactivated insulin preparations, and then fell. The depression of blood ketones with the inactivated material is probably not greater than would be expected on the basis of the residual hypoglycemic activity of the prepara-

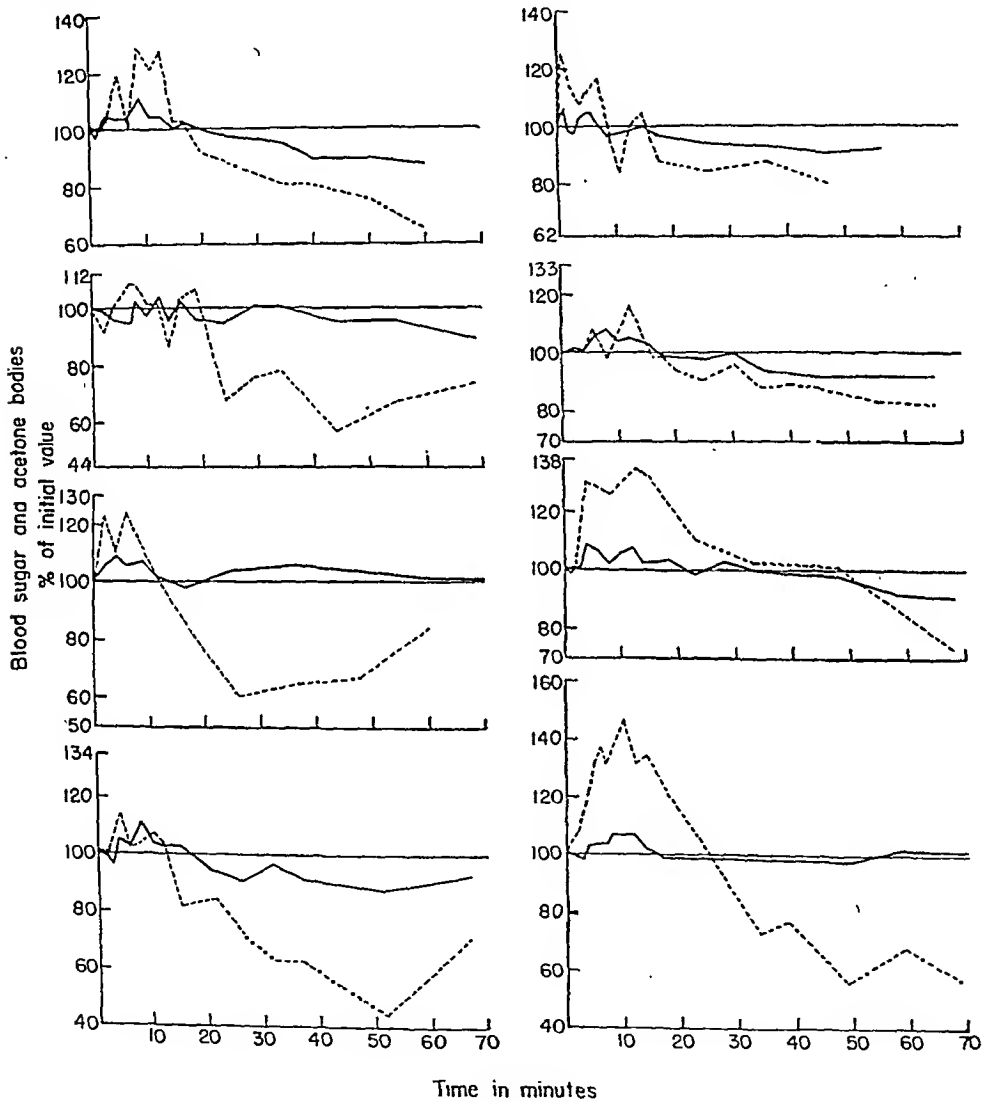


Fig. 3. COURSE OF BLOOD SUGAR and acetone bodies in 8 experiments following intravenous injection of 0.1 mgm/kilo of cysteine-inactivated amorphous insulin. Solid line: sugar; broken line: acetone bodies.

tions. Although the magnitude of the initial hyperketonemic response was variable, the direction and time relationships of the ketone curves followed the changes in blood glucose very closely. This finding is consistent with what has been described concerning the changes in liver glycogen under these circumstances. DeDuve, Hers and Bouchaert (20) have shown that glycogen levels fall during the first few minutes after the injection of ordinary insulin preparations. Recently Sutherland and Cori

TABLE 3. VALUES FOR BLOOD SUGAR AND ACETONE BODIES FOLLOWING INTRAVENOUS ADMINISTRATION OF INACTIVATED AND INTACT AMORPHOUS INSULIN IN PANCREATECTOMIZED DOGS

INACT. AMORPHOUS INSULIN, 0.1 MG./KILO EXP. NO.		INITIAL LEVEL		MAXIMUM		LOWEST LEVEL ¹	
		Sugar	Acetone bodies	Sugar	Acetone bodies	Sugar	Acetone bodies
		mgm. per 100 cc.		% of initial value			
1		250	2.08	111	129	88	66
2		285	2.81	104	106	69	58
3		314	8.75	107	124	98	60
4		282	1.73	105	114	91	46
5		237	1.76	105	117	91	80
6		270	3.60	108	116	92	82
7		267	0.585	108	134	91	73
8		231	2.86	107	146	99	57
INTACT AMORPHOUS INSULIN							
Exp. No.	Dose U/kilo						
1	1	320	2.75	109	115	72	22
2	3	220	2.89	105	109	74	43
3	4	223	0.819	106	195	48	34

¹ In 60 to 70 minutes.

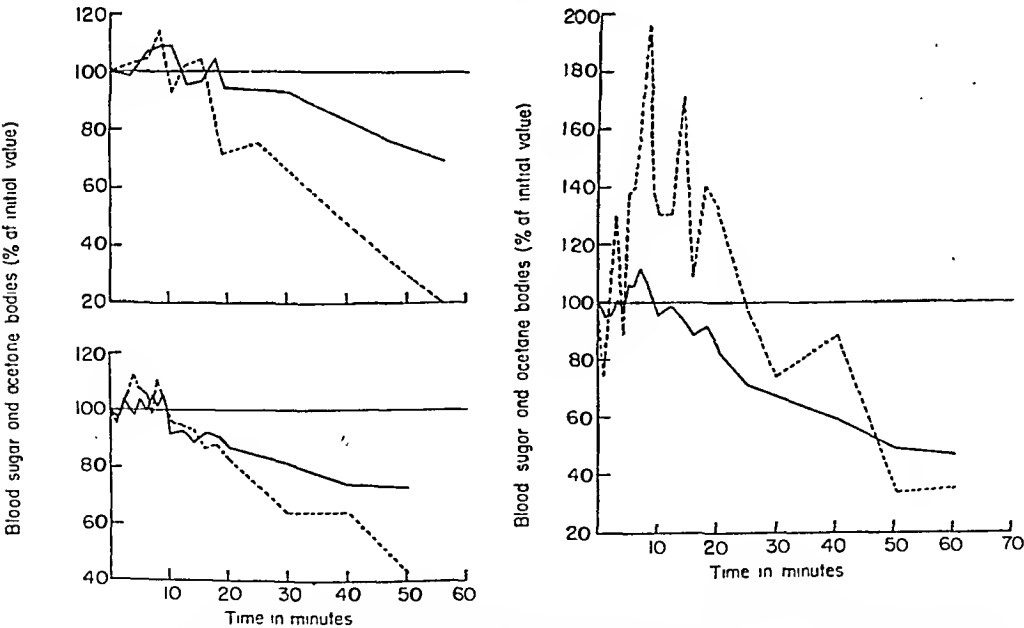


Fig. 4. COURSE OF BLOOD SUGAR and acetone bodies following intravenous administration of 1 (*upper left*), 3 (*lower left*) and 4 (*right*) U/kilo of intact commercial (amorphous) insulin. *Solid line*: sugar; *broken line*: acetone bodies.

(21) demonstrated both glycogenolysis and glycogen formation in liver slices in the presence of ordinary insulin preparations. Cysteine-inactivated insulin caused only glycogenolysis. Both of these groups, furthermore, found the 'Novo' insulin to have no glycogenolytic activity. In line with the principles expressed by Mirsky (22)

and others concerning the importance of liver glycogen depletion in the pathogenesis of ketosis, one would expect these changes in blood glucose in our experiments to be reflected in the blood ketone levels. The rapidity with which the response occurs however is quite surprising.

It seemed important to decide whether the hyperglycemic principle was operating as an insulin antagonist, either by neutralizing insulin or by blocking its entrance into some biochemical system. It is likely, however, that if this were the case, there should be some dose in which it would protect against the convulsive effect of insulin. This we have been unable to demonstrate. The absence of such an effect would seem to preclude the possibility that this substance is a factor responsible for insulin resistance or for the high insulin requirements which characterize some of the experimental situations described above. It is consistent on the other hand with the theory that the hyperglycemic factor acts only on the hepatic glycogen

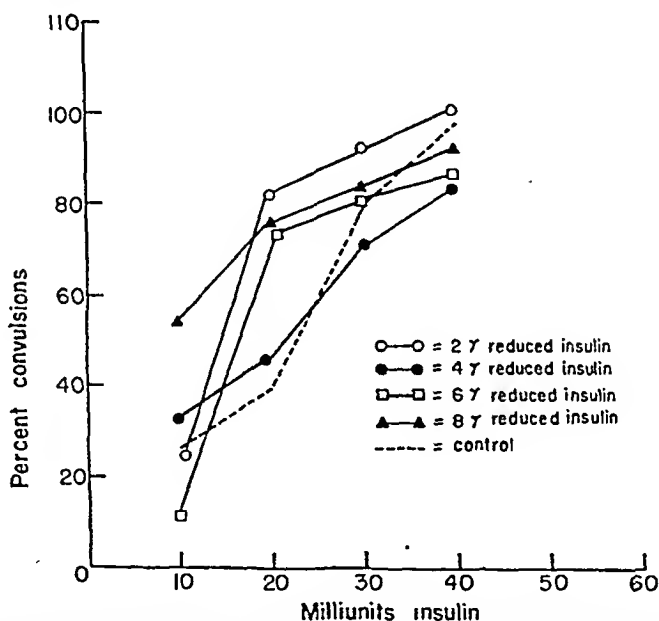


Fig. 5. GRAPH SHOWING RELATIONSHIP between insulin dosage and percentage convulsions in mice (control) and same relationship in groups in which varying amounts of inactivated insulin were given in addition.

reserves. Since these are always mobilized to the maximum possible extent in the face of impending hypoglycemic convulsions, a substance which acts merely to release liver glycogen would not be expected to exhibit a protective effect.

If the pancreas does produce a hyperglycemic hormone there arises the question of how the deficiency of this substance alone would manifest itself. A possible answer to this question was given by Dr. S. H. Armstrong of Boston (23) who suggested that the clinical entity representing such deficiency might be von Gierke's disease. The existence of hypoglycemia and inability to release glycogen would conform to this hypothesis.

SUMMARY

1. Numerous investigators have described hyperglycemic substances derived from pancreas. Commercial insulin possesses a hyperglycemic property which is not

destroyed when its hypoglycemic activity is inactivated by various methods. The existence of a hyperglycemic principle of pancreatic origin is of interest because evidence obtained from alloxan-diabetic dogs and other preparations suggest that the pancreas may produce a hormone which opposes insulin. The experiments discussed in this report deal with the hyperglycemic activity of insulin.

2. Experiments were performed to determine the effect of the hyperglycemic material which remains after cysteine inactivation of amorphous and crystalline insulin preparations. Both of these preparations gave very similar results with respect to magnitude and duration of hyperglycemia in normal dogs.

3. In pancreatectomized dogs not treated with insulin the hyperglycemic response was small and the blood acetone body levels rose simultaneously with the blood sugar following the intravenous administration of both intact and inactivated insulin.

4. Inactivated insulin in various doses does not protect mice against insulin-induced convulsions.

5. The data support the conclusion that the hyperglycemic principle of insulin influences the blood sugar and blood ketone levels only through its action on the hepatic glycogen reserves. It does not appear to neutralize the hypoglycemic action of insulin. Whether or not this principle has a normal function as an internal secretion of the pancreas is still problematical.

The authors are appreciative of the technical assistance given by J. W. Thomas, Chief Pharmacist's Mate, USN. They are indebted to Eli Lilly and Company for the dry zinc-insulin crystals which were used.

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RENAL GLUCOGENESIS IN THE EVISCERATED DOG¹

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THE kidney has been found to be a source of blood sugar (1). Since this phenomenon has been demonstrated only in the rat, it has seemed desirable to examine other species for its presence. To this end we have studied the concentrations of sugar in arterial and renal vein blood from the eviscerated dog.

PROCEDURES

The dogs were eviscerated by a modification of the glass cannula method (2). The intramesenteric organs were removed through a midventral incision after dividing the mesenteric root and lesser omentum and severing the following structures between clamps: colon opposite the sacral promontory, inferior mesenteric artery, superior mesenteric artery, structures entering the porta hepatis, and stomach just below the cardia. The stumps of these structures were ligated and the clamps removed. The attachments of the liver were then divided so that it remained connected to the animal only at the places where the vena cava entered and left it. A 5 cm. length of the vena cava midway between the junction of the left renal vein and the confluence of the common iliac veins was next dissected free. It was usually necessary to ligate and sever one of the lumbar veins in doing this. Loose ligatures were placed at either end of this freed segment. When an assistant lifted the vessel by means of these, they served to prevent blood from entering the intervening section. This was then opened by a V incision. A glass cannula was inserted toward the heart. Briefly relaxing the tension on the proximal lifting ligature allowed the cannula to slip past. The distal end of this cannula was then tied in place by a ligature around the vena cava at its entrance into the liver. The hole in the vena cava was closed by a second cannula that was tied in place by the lifting ligatures. The vena cava was next tied snugly to the proximal end of the first cannula by a heavy ligature at the place where it emerges from the liver. The liver and the transversing segment of vena cava were now dissected away to leave the stumps of the vena cava connected by the cannula. The cannula used to replace the segment of vena cava transversing the liver should be about 7 cm. long and 1 cm. in diameter for a 15-kgm. dog. The cannula used to close the opening in the vena cava may be somewhat shorter. The ends of the cannulas should be beveled and fire-polished. There should be an encircling ridge at either end to prevent the ligatures from slipping off. The cannulas in these experiments were made of Pyrex glass and coated with

Received for publication April 12, 1948.

¹ Aided by a grant from the Committee on Scientific Research of the American Medical Association.

Dri-Film (3). There was no difficulty with clot formation. The animals were anesthetized with sodium pentobarbital given intraperitoneally.

Arterial and left renal vein blood samples were drawn simultaneously and treated with 2 mgm/ml. of potassium oxalate. Copper tungstate filtrates were prepared (4). Portions of some of these were incubated for 45 min. at 30°C. with 0.2 gram/ml. of baker's yeast that had just been washed ten times with distilled water (5). Both the fermented and unfermented filtrates were analyzed in duplicate or triplicate for sugar by a copper reduction method (6). The values obtained for the unfermented filtrates are designated total sugar. The differences between these values and those obtained on the fermented filtrates are designated fermentable sugar and probably approximate fairly closely the blood glucose concentration. Copper tungstate filtrates of glucose solutions and of blood with added glucose were fermented as a check to show that these filtrates are fermented adequately by the yeast used. The quality of the yeast was checked for each group of analyses by running parallel fermentations on standard glucose solutions and distilled water.

The animals were mongrel dogs of either sex and undetermined previous history. They were operated in the postabsorptive state, usually after fasting from the previous day. Several animals that had been allowed to fast longer withstood the evisceration so poorly that samples were not obtained.

RESULTS AND DISCUSSION

The results are shown in table 1. Consideration of the values shown in the column headed 'Excess in Renal Vein, mgm/100 ml.' shows that more sugar, either total or fermentable, was usually found in the renal vein after evisceration. To determine whether or not this result might be a reflection of the random distribution of experimental error these values were analyzed statistically by the method for testing the significance of the mean of a unique sample (7, 8). The mean for the post-evisceration total sugar values is 3.07 mgm/100 ml. with a standard deviation of 0.587 mgm/100 ml. giving a value for t of 5.23 and for P of less than 0.001. This indicates that the random distribution of error cannot explain the observed excesses of total sugar and that therefore these probably represent real excesses present in the renal vein blood. The corresponding values for the fermentable sugar were mean, 5.43 mgm/100 ml.; standard deviation, 0.921 mgm/100 ml.; t , 5.89; and P less than 0.001. The post-evisceration samples to be analyzed for fermentable sugar were in part selected at random, in that in some instances not enough filtrate was available for the procedure, and in part systematically, in that some samples were not analyzed if the analysis for total sugar showed that the excess of sugar in the renal vein was so small that it was likely to be masked by the analytical error inherent in the determination of fermentable sugar. The statistical result then indicates only that the observed values are not explicable on the basis of the random error peculiar to the determination of fermentable sugar. It follows then that in the filtrates from this selected group of pairs of samples there was more fermentable sugar in those from the renal vein than in those from the arterial blood. Since the greater part of the experimental error probably pertains to the analysis for sugar, this supports the conclusion that at times there is an actual excess of fermentable sugar in the renal vein as compared with the aorta.

TABLE 1

ANIMAL NO. AND WT.	MIN. AFTER EVISCEATION	ARTERIAL BLOOD SUGAR	RENAL VEIN BLOOD SUGAR	EXCESS IN RENAL VEIN	EXCESS IN RENAL VEIN, % OF ARTERIAL
		mgm. %	mgm. %	mgm. %	
1 10.8 kgm.	-10 120 125 240 died	69.5 (66) 22 (16.5) 26 (19)	69.5 (66) 25 (20.5) 32 (20.5)	0 (0) 3 (4) 6 (1.5)	0 (0) 13.6 (24.2) 23.1 (7.9)
2 8.4 kgm.	120 190 died	30 (19)	33 (19.5)	3 (0.5)	10 (2.6)
3 11.1 kgm.	-15 140 170 died	99.5 (92.5) 18 (5)	88 (79) 25.5 (13)	-11.5 (-13.5) 7.5 (8)	-11.6 (-14.6) 41.7 (160)
4 16.6 kgm. ♂	-15 120 195 died	84 (75) 50 (37)	79 (66.5) 56 (43.5)	-5 (-8.5) 6 (6.5)	-5.9 (-11.3) 12 (17.6)
5 ¹ 14.6 kgm. ♂	-10 150 325 died	79 (75) 41.5	77.5 (75) 47.5	-1.5 (0) 6	-1.9 (0) 14.4
6 ^{1, 2} 20 kgm. ♂	80 120 150 180 210 240 died	82.5 65 45.5 (42) 43.5 32	81 60 50 (43.5) 52 39	-1.5 -5 4.5 (1.5) 8.5 7	-1.8 -7.7 9.9 (3.6) 19.5 21.9
7 ^{1, 3} 10 kgm. ♀	122 162 202 217 died	67 (63.5) 61 28.5	69 (65.5) 63 20	2 (2) 2 -8.5	3.0 (3.1) 3.3 -29.8
8 ^{1, 3} 17.4 kgm.	-13 64 105 135 165 180 died	82 58 (52) 51 (45) 43 31 (19)	78 70 (65.5) 55 (48) 48.5 41.5 (30.5)	-4 12 (13.5) 5 (3) 5.5 10.5 (11.5)	-4.9 20.7 (25.9) 9.8 (6.7) 12.8 33.9 (60.5)
9 ¹ 20 kgm.	85 120 135 died	42 (34) 29.5 (23)	49.5 (45) 37.5 (30.5)	7.5 (11) 8 (7.5)	17.8 (32.4) 27.1 (32.6)
10 ^{1, 4} 20.2 kgm. ♂	90 120 150 190 220 300 died	82 (80) 74 (72.5) 68 (62.5) 50 42	83.5 (81) 76.5 (75.5) 69 (63.5) 52.5 44.5	1.5 (1) 2.5 (3) 1 (1) 2.5 2.5	1.8 (1.2) 3.4 (4.1) 1.5 (1.6) 5 6.0

TABLE 1—Continued

ANIMAL NO. AND WT.	MIN. AFTER EVisCERATION	ARTERIAL BLOOD SUGAR	RENAL VEIN BLOOD SUGAR	EXCESS IN RENAL VEIN	EXCESS IN RENAL VEIN, % OF ARTERIAL
		mgm. %	mgm. %	mgm. %	
11 ^{1, 5}	90	50.5	50	-.5	-1.0
	120	35.5	37.5	2	5.6
	180	25.5	25.5	0	.0
17.2 kgm. ♂	220	17.5	20.5	3	17.1
	223	16.5	17.5	1	6.1
	260	11 (8)	16 (14.5)	5 (6.5)	45.5 (81.3)
	270	died			
12 ^{1, 6}	60	100	101	1	1
	73	86	89	3	3.5
	78	.5 mgm. epinephrine hydrochloride I.V.			
14.1 kgm. ♂	83	80	82.5	2.5	3.1
	93	75.5	78.5	4	5.3
	98	66.5 (59)	74.5 (68.5)	8 (9.5)	12.0 (16.1)
	140	58	60	2	3.4
	170	44	52.5	8.5	19.3
	200	27.5 (21)	35.5 (27.5)	8 (6.5)	29.1 (30.9)
	215	.5 mgm. epinephrine hydrochloride I.V.			
	260	died			
13 ^{1, 6}	94	69.5	70	0.5	0.7
	106	6.4	65	1	1.6
	118	1 mgm. epinephrine hydrochloride I.V.			
24 kgm. ♂	123	72	61	-11	-15.3
	133	51.5	53.5	2	3.9
	150	45 (36)	52 (46)	7 (10)	15.5 (27.8)
14 ^{1, 6}	85	71	72.5	1.5	2.1
	105	66.5	66	-.5	-.8
	120	60.5	60	-.5	-.8
	135	57	58	1	1.7
	150	56	57	1	1.8
	165	50.5	51.5	1	2.0
	180	47 (43.5)	49 (42)	2 (-1.5)	4.3 (-3.4)
	195	38 (33.5)	46 (41)	8 (7.5)	21.0 (22.4)
	200	died			

Values shown in parentheses are fermentable sugar. The others are total sugar.

¹ A cannula connected to a manometer was tied into the ipsilateral (left) ureter. The system was filled with normal saline till the level in the open arm stood at 30 to 40 cm. above the kidney.

² Blood samples taken through small bore polyvinyl chloride catheters placed in an artery and the renal vein at the time of evisceration.

³ Blood samples from renal vein taken through a catheter or needle left in place after evisceration.

⁴ Blood samples from renal vein and aorta taken through needles placed at end of evisceration procedure.

⁵ The first two pairs of samples taken as described in footnote 3. Last three samples taken after first painting the site of puncture on the renal vein with 0.1% nupercain in saline.

⁶ Left kidney was dissected free from the rest of the organism except for renal artery and vein. These were repeatedly painted with 0.1% nupercain in saline.

Nonglucogenic processes such as the excretion of urine and variations in renal volume and sugar content may account for some increase in the sugar concentration observed in the renal vein (1). In some of these studies the excretion of urine was prevented by back pressure in the ureter. Because of the richness of renal bloodflow the variations in volume and sugar content can only account for brief increases in renal vein sugar concentration. An attempt was made to rule out these short-term variations by repeated sampling and avoiding stimulation associated with the sampling process that would be likely to initiate them. None of these procedures seemed to eliminate the excesses of sugar found in the renal vein. This seems to support the conclusion that these nonglucogenic processes do not account for the observed excesses. Further information on this point can be obtained by considering the values shown in table 1 in the column headed 'Excess in Renal Vein, % of Arterial'. Consideration of the possible nonglucogenic processes that might elevate the renal vein sugar shows that the increases they would effect would be proportional to the arterial blood sugar level (1). By converting the observed increases into percentages of the corresponding arterial values it is possible to make some estimate as to whether or not the nonglucogenic processes would be likely to effect increases of comparable magnitude. Statistical treatment of the postevisceration values shows a mean of 8.86 per cent for the total sugars with a standard deviation of 1.685 per cent, giving a value of t of 5.25 and a value of P of less than 0.001. The nonglucogenic processes would have to account for an increase of over 5.36 per cent to vitiate the conventional significance attached to these values. For the reasons discussed apropos the rat (1) it seems unlikely that the nonglucogenic processes cause an increase of this magnitude and that therefore these values support the interpretation that the kidney added substances to the blood measurable as total sugar after evisceration.

SUMMARY

The concentration of sugar was found to be greater in renal vein than in arterial blood in the eviscerated dog.

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EFFECT OF CASTRATION AND ANDROGENS ON BODY AND ORGAN WEIGHTS, AND THE ARGINASE AND PHOSPHATASES OF KIDNEY AND LIVER OF THE MALE SYRIAN HAMSTER¹

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IN PREVIOUS reports the effect of castration and various androgens on the weights, and the arginase and 'alkaline' and 'acid' phosphatase activities of the kidney and the liver of the mouse and the rat have been reported (cf. 1, 2 for review). These studies now have been extended to the hamster.

PROCEDURE

Male Syrian hamsters were castrated at 45 to 75 grams body weight; one month later the various treatments were begun. In one series of experiments treatment was begun at time of castration.² The animals were kept in groups of 4 to 7 in rabbit cages containing wood shavings. Three different diets were used: *a*) Rockland Rat diet, *b*) Rockland guinea pig diet with vitamin C and *c*) a prepared diet² composed of cornstarch 25, whole wheat flour 20, confectionery sugar 20, whole milk powder 30 and powdered alfalfa. The diets did not affect the results. The animals were weighed two to three times per week throughout the experimental period.

The androgens were administered either by daily subcutaneous injection or by the subcutaneous implantation of pellets of the pure steroids.³

The hamsters were placed in clean cages without food 24 hours before autopsy. They were anesthetized by an intraperitoneal injection of 0.10 to 0.15 ml. of dial-urethane and then bled by cutting the blood vessels in the neck. The organs were removed and weighed on a Roller-Smith torsion balance. The kidneys and approximately one gram of the liver were placed in individual heavy walled pyrex tubes containing 5 ml. cold redistilled water and homogenized for the enzyme determinations (3-6).

RESULTS

Body weight. Neither castration nor the daily injection of 1 mgm. of testosterone propionate for 20 or 60 days produced any noteworthy changes in the body

Received for publication April 28, 1948.

¹ This investigation was supported by grants from Ciba Pharmaceutical Products, Inc. Part of these data has been reported in the Josiah Macy Jr. Foundation Conference on Metabolic Aspects of Convalescence. Thirteenth meeting, June 10-11, 1946.

² The animals of this experiment were from an experiment by Dr. Paul Keyes of the Dental Research group for studies on dental caries.

³ The steroids and dial urethane were generously provided by Ciba Pharmaceutical Products, Inc.

weight of the hamsters (table 1). However, when 3 pellets of 14-15 mgm. each of testosterone or 17-methyltestosterone were implanted in each animal for 20 days

TABLE 1. EFFECT OF CASTRATION AND INJECTION OF TESTERONE PROPIONATE (T.P.) ON BODY AND ORGAN WEIGHTS OF MALE HAMSTERS

	NO.	ANDROGEN INJECTED	BODY WEIGHT		SEM. VES. + PROS.	KIDNEYS	LIVER
			End	Change			
I. 20-day experiment							
		mgm./day	gm.	gm.	% ¹	% ¹	% ¹
Castrate.....	9 ²	—	97	+5	(83)	(709)	(2390)
Normal.....	6	—	92	+11	+700	0	+2
Castrate + T.P.....	10 ²	1	96	+5	+1410	+14	+14
II. 60-day experiment							
Castrate.....	3	—	114	+17	(56)	(724)	(2670)
Normal.....	6	—	113	+16	+1400	-5	+5
Castrate + T.P.....	5	1	105	+13	+3460	+13	-2

¹ % Change from the average values of the controls which are given in parentheses as milligrams.

² Four of the castrate and 5 of the T.P.-treated hamsters were maintained on the Rockland guinea pig diet with vitamin C. The other hamsters were maintained on the Rockland rat diet. No significant differences were noted.

TABLE 2. EFFECT OF CASTRATION AND PELLETS OF ANDROGENS ON BODY AND ORGAN WEIGHTS OF MALE HAMSTERS

	NO.	ANDROGEN ABSORBED	BODY WEIGHT		SEM. VES. + PROS.	KIDNEYS	LIVER
			End	Change			
III. 20-day experiment ²							
		mgm./day	gm.	gm.	% ¹	% ¹	% ¹
Castrate.....	7	—	100	10	(116)	(743)	(2698)
Castrate + testos- terone ³	6	0.72	92	2	+978	+1	0
Castrate + 17-methyl- testosterone ⁴	6	0.50	74	-16	+765	-5	-16
IV. 140-day experiment ²							
Castrate.....	5	—	140	81	(46)	(612)	—
Normal.....	5	—	119	68	+2820	+1	—
Castrate + T.P. ⁵	5	0.15	104	56	+4250	-6	—

¹ % Change from the average values of the controls which are given in parentheses as milligrams.

² These hamsters were maintained on the Rockland Rat diet.

³ These animals were maintained on the prepared diet.

⁴ Each animal was implanted subcutaneously with 3 pellets of 14-15 mgm. each.

⁵ The pellet, 24-26 mgm., was implanted at time of castration, 48± grams body weight.

there was a definite retardation in the body weight gain which was more marked with 17-methyltestosterone (table 2). Furthermore, extension of the duration of the

TABLE 3. EFFECT OF CASTRATION AND INJECTION OF TESTOSTERONE PROPIONATE (T.P.) ON KIDNEY AND LIVER ENZYMES OF MALE HAMSTERS¹

	NO.	KIDNEY			LIVER		
		Arginase	Phosphatases		Arginase	Phosphatases	
			Alkaline	Acid		Alkaline	Acid
I. 20-day experiment							
Castrate.....	9	% ² (59)	% ² (31)	% ² (13.3)	% ² (9.830)	% ² (2.7)	% ² (15)
Normal.....	6	-17	-12	-4	+20	-10	-6
Castrate + T.P.....	10	-20	+32	+2	+3	-18	-5
II. 60-day experiment							
Castrate.....	3	(59)	(19)	(11.1)	(11,030)	(2.2)	(14)
Normal.....	6	-34	+42	-2	-5	-18	-12
Castrate + T.P.....	5	-37	+68	-4	-29	-14	-8

¹ See footnotes of table 1 for details of experiments.² % Change from the average values of the controls which are given in parentheses as units/gram of tissue.TABLE 4. EFFECT OF CASTRATION AND PELLETS OF ANDROGENS ON KIDNEY AND LIVER ENZYMES OF MALE HAMSTERS¹

	NO.	KIDNEY			LIVER		
		Arginase	Phosphatases		Arginase	Phosphatases	
			Alkaline	Acid		Alkaline	Acid
I. 20-day experiment							
		% ²	% ²	% ²	% ²	% ²	% ²
Castrate.....	7	(56)	(24)	(13.8)	(14,700)	(2.6)	(15)
Castrate + testosterone.....	6	-14	+62	+7	-9	+19	-1
Castrate + methyltestosterone.....	6	-11	+68	+6	+1	0	+4
IV. 140-day experiment							
		(51)	(33)	(8.6)	(7,310)	(3.6)	(13)
Castrate.....	5	(51)	(33)	(8.6)	(7,310)	(3.6)	(13)
Normal.....	5	-18	+28	-11	+5	-25	-8
Castrate + T.P.....	5	-33	+41	+9	+7	-44	+5

¹ See footnotes of table 2 for details of experiments.² % Change from the average values of the controls which are given in parentheses as units/gram of tissue.

experiments to 140 days resulted in an extensive and grossly obvious deposit of fat in the castrated hamsters which was prevented in the animals implanted with a 24-26 mgm. pellet of testosterone propionate.⁴

⁴ These data will be reported in detail elsewhere by Dr. Paul Keyes.

Seminal vesicles and prostates. In every experiment (tables 1 and 2) these organs were stimulated by androgen treatment to a size greater than that present in the normal animals of comparable age.

Kidneys. Castration produced no significant change in kidney weight. Furthermore, the administration of the androgens produced insignificant changes which varied from -6 to +14 per cent (tables 1 and 2).

Livers. No remarkable changes in weight were observed as a result of castration or androgen treatment (table 1 and 2).

Kidney enzymes. In every experiment (tables 3 and 4) there was a small increase in arginase as a result of castration. The various androgen treatments restored this increase to that of the normal level. The 'alkaline' phosphatase was decreased after castration (tables 3 and 4) except in the first series of experiments (table 3). The androgens increased the enzyme to that of the normal value. The 'acid' phosphatase was not significantly altered by either castration or any of the various treatments (tables 3 and 4).

Liver enzymes. None of the enzymes were significantly altered by castration or the various androgen treatments (tables 3 and 4).

DISCUSSION

In the hamster, the only internal organs that are affected by castration or androgens seem to be the accessory sex organs. The liver and kidney are not changed in size under the various treatments. The lack of effect on the liver is in general agreement with that observed in the other species (1, 2) but in some instances the liver of the rat (7, 8) and mouse (unpublished) is increased to a small degree by androgen treatment.

The lack of effect of either castration or androgen treatment on the kidney of the hamster is in marked disagreement to that observed in the mouse (1; cf. 1, 2) and to a lesser extent in the rat (cf. 1, 2) but is in agreement with that observed in the guinea pig (unpublished).

The lack of effect of either castration or androgen treatment on the liver enzymes is the same as observed in the other species (cf. 1, 2). Thus, it seems that in the hamster as in the other species, liver arginase and also the phosphatases either are not involved in the mechanism of the protein anabolic effects of the androgens or else the degree of change in the metabolic processes is not sufficient to necessitate a change in the activities of these enzymes.

The decrease in kidney arginase after castration and restoration to normal with androgen treatment is similar to the observations in the mouse except that in the mouse the decrease occurred only as the kidney was restored to normal by androgen stimulation. When the dose of androgens was increased there was a progressive increase in the arginase independently of the size of the kidney. The hamster, on the other hand, does not show this second response in spite of the fact that large doses of androgens including 17-methyltestosterone, the most potent stimulator of kidney arginase, were used. Indeed 1 mgm/day of testosterone propionate will produce a very marked increase of this enzyme in the rat kidney within a few days. The possibility of dietary intake as a factor has been at least partially ruled out since

three different diets were used and one of these was the same as that used for rats and mice while another was the same as that used for guinea pigs. The possibility of an as yet unrecognized dietary factor essential for the kidney arginase response, however, may exist.

The increase in 'alkaline' phosphatase and no effect on 'acid' phosphatase are in agreement with these observations in the rat (9, unpublished) and guinea pig kidney (unpublished).

These variations in response of different species to castration and androgen treatment are of special and immediate significance since they are reflections of differences in mechanism of metabolic processes among the various species.

SUMMARY

Castration caused a decrease in the size of the seminal vesicles and prostates but no change in the kidney or liver. The administration of testosterone propionate by injection or by the subcutaneous implantation of a pellet for 20 and 140 days increased the seminal vesicles and prostates of castrated hamsters to greater than normal but did not affect the size of the kidney or liver. Pellets of testosterone and 17-methyltestosterone implanted subcutaneously for 20 days produced similar responses.

The arginase of the kidney increased as a result of castration and decreased to normal with the various androgen treatments. The 'alkaline' phosphatase on the other hand decreased after castration and was restored to normal with androgen treatment. The 'acid' phosphatase of the kidney and the arginase and phosphatases of the liver were not affected by castration or by the androgen treatment.

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PARROT FISH THYROID EXTRACT AND ITS EFFECT UPON OXYGEN CONSUMPTION IN THE FISH, BATHYSTOMA

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THE physiology of the teleost thyroid still awaits clarification. While several investigators have tried (1-4), no one has succeeded as yet in changing the oxygen consumption of teleost fish upon the administration of thyroid hormone of mammalian origin. Further, there is no agreement in the literature on the effect of mammalian thyroid hormone on other physiological functions of fish, such as growth and differentiation. It hardly seems necessary to recapitulate this literature here since it has been thoroughly reviewed elsewhere (3, 4).

With the advent of anti-thyroid drugs such as thiourea and thiouracil, a new approach to the problem was possible. The results to date have been scanty. Goldsmith *et al.* (5) reported a retardation of growth in platys grown in thiourea solutions. However, Matthews and Smith (4) were unable to show any change in the oxygen consumption of adult *Fundulus* after daily injections of thiourea. These results, while not necessarily contradictory, indicate the desirability of further investigation.

In the past, students of the physiology of the fish thyroid have uniformly used mammalian thyroid extracts. We have felt for sometime that such preparations might not be optimal. But until recently, because of the diffuse nature of the thyroid tissue in most fishes, the difficulties in obtaining an adequate supply of teleost thyroid have been too great to permit the manufacture of extracts of teleost origin. However, Matthews (6) has recently described in the Bermuda parrot fish a large and discrete thyroid gland. Since this fish (weighing up to 30 pounds) is not too difficult to obtain in Bermuda waters and since one fish will yield about 29 mgm. of dried thyroid tissue per kilogram, a plentiful supply of glandular tissue has become available for extraction.

The probability that an active thyroid extract could be prepared from such tissue was further strengthened by the observation of Matthews and Smith (7) that iodine is concentrated in the thyroid gland of the parrot fish (*Sparisoma* sp.). This was done by injecting the radioactive isotope I^{131} into small parrot fishes, removing the glands and applying them to unexposed x-ray plates. Such a procedure yielded autographs of the gland after several days of exposure.

METHODS

Preparation and injection of extract. Extracts were prepared by macerating dried or fresh thyroid tissue in sea water. Drying was done in an oven at 50°C. for 24 hours, or in acetone. No

Received for publication April 15, 1948.

attempt at purification was made. Extracts were also prepared from dried brain, muscle and liver of parrot fish. The common Bermuda white grunt (*Bathystoma* sp.) was used as the test animal. All extracts were given intra-abdominally with a hypodermic syringe. Care was taken not to injure the swim bladder or other vital organs.

Respiration chambers. For several days prior to injection all fish were kept in respiration chambers, maintained at a constant temperature. The chambers were of two sizes, one with a capacity of 250 cc. for small fish and the other with a capacity of 500 cc. for larger ones. The grunts fitted comfortably into the chambers and were allowed a certain amount of restricted movement. As a rule they adapted themselves quickly to the new environment and after the first day remained relatively motionless, except for an occasional movement of gill or fin.

A continuous flow of water was maintained through each chamber; the rate of flow over any appreciable period was constant, varying from 50 to 75 cc. per minute.

Water temperature was kept constant by means of a constant temperature bath and a coil. It was not possible, however, to perform all experiments at the same temperature. Experiments were begun in March, with the bath temperature set at 20°C. As the season advanced it was necessary to increase the temperature by 0.5 to 1.0°C. every two or three weeks to prevent too great a difference between the bath temperature and the outside water temperature. When the experiments were concluded late in May, the bath temperature stood at 25°C.

The oxygen content of the incoming sea water varied from 5.71 cc. to 4.67 cc. per liter. The trend was downward as the outside water warmed, but the change was not consistent from day to day since variations, while small, were random and impossible to predict in advance. The oxygen content of the outflow water varied with the size and the metabolic state of the fish, but it was never diminished by more than 0.75 cc. per liter less than that of the inflow.

No fish stayed in the chamber more than 19 days. The average period was 9 days. None of the animals was fed during the experimental period and all lost weight at an average rate of about 0.2 gram a day. About four fifths of the grunts placed in the chambers survived. They were killed at the end of the experiment to determine their sex. The remaining one fifth died before removal from the chambers. Only two animals (*nos.* 2 and 24, table 1) which died before the end of the experiments were included in calculating the final results. The most common cause of death was stoppage of the flow of water through the chambers, due to failure of the water supply or plugging of the tubing with debris.

Collection and analysis of samples. Samples of the inflow water to all chambers, as well as samples of the outflow from each individual chamber, were taken daily in the morning. The dissolved oxygen in each sample was determined by the Birge-Juday (1911) modification of the Winkler method. Oxygen consumption for each fish at the time of sampling was determined from the difference in the oxygen tension of the inflow and the outflow water, and from the rate of flow of water through the chamber. Results were expressed in cubic millimeters of oxygen used by each fish per minute rather than in cubic millimeters per gram per minute. The latter expression was felt to be relatively meaningless since no correction could be applied for weight loss during the experiment or for error in weighing. All injections were made after the sampling for the day had been completed. Consequently the fish were undisturbed for approximately 24 hours after each injection before another determination of oxygen consumption was made. Previous experience had shown us that handling the fish for injection produced no change in oxygen consumption lasting more than one or two hours.

RESULTS

The results of the experiments are summarized in the following charts and tables. Figure 1 shows the daily oxygen consumption of three different white grunts kept in the chambers for varying lengths of time and treated in different ways. *Fish A* was left undisturbed for 12 days. After the first day the oxygen consumption varied in a seemingly random fashion, between 38 mm.³ per minute and 66 mm.³ per minute, with an average of 53.4 mm.³. This pattern may be considered typical of all untreated fish. Almost without exception oxygen consumption was highest on the

first day in the chamber and subsided on the second or third day to a level which, within the limits of variability, was constant from then on. This is in contrast to the behavior of *Fundulus*, where oxygen consumption progresses steadily downward while the fish are confined in respiration chambers (4). The daily variations seen in white grunts probably can be attributed to differences in activity. Spoor (5) has recently emphasized the variability in activity among goldfish and its effect on oxygen consumption. In our experiments the fish were usually left in the chambers for three to five days before injection and thus were given ample time to adjust themselves to their surroundings.

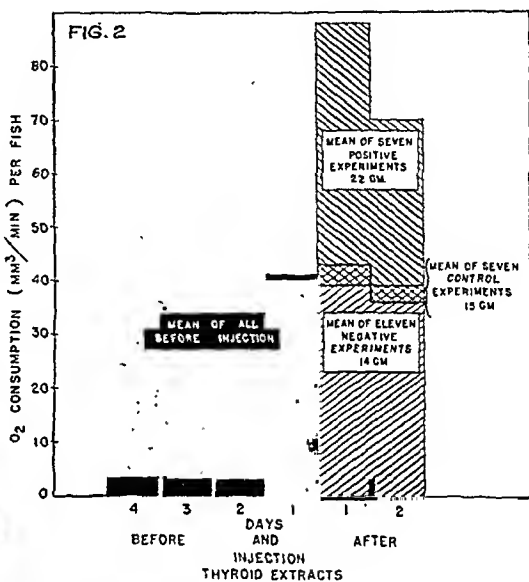
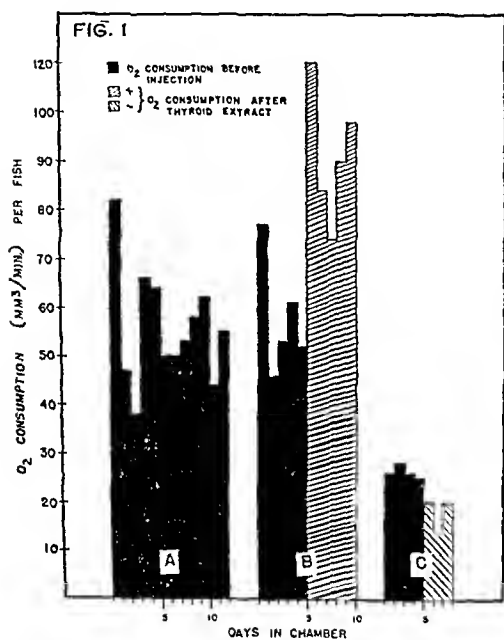


Fig. 1. OXYGEN CONSUMPTION in the white grunt, *Bathystoma* sp. A. Control. B. Positive response after injection of parrot fish thyroid extract. C. Negative response after injection of parrot fish thyroid extract.

Fig. 2. OXYGEN CONSUMPTION in the white grunt, *Bathystoma* sp., before and after injection of parrot fish thyroid extract.

The grunts which were injected with parrot fish extracts may be divided into two groups: 1) those which showed an unmistakable rise in oxygen consumption after injection (such as B, fig. 1), and 2) those which did not (such as C, fig. 1). A little less than one half of the fish injected fell into the first group. Hereafter they will be referred to as animals giving a positive response to thyroid extract, and those in the second group will be referred to as animals giving a negative response. In the second group are included those animals which obviously gave no response (such as C, fig. 1), as well as those which, while giving a rise in oxygen consumption, did not give one above the range that could be attributed to random variation.

In table 1 the data are summarized in tabular form, grouping the animals in three categories: 1) those which showed a positive response to thyroid extract, 2) those which showed a negative response to thyroid extract, and 3) those which were injected with extracts of tissues other than thyroid gland, such as brain, muscle and

TABLE 1. OXYGEN CONSUMPTION IN WHITE GRUNTS BEFORE AND AFTER INJECTION OF PARROT-FISH THYROID GLANDS

FISH NO. & SEX	INITIAL WT.	EXTRACT	DAYS								% CHANGE FROM DAY BEFORE INJ.
			Before injection				After injection				
			4	3	2	1	1	2	3	4	

gms.			O ₂ in mm. ³ /min.							
Negative responses to thyroid extracts										
1m	10	thyroid	26	28	26	25	20	14	20	-20
2f	15	thyroid		27	33	28	34	Died		21
3f	11	thyroid		40	32	40	59	43	37	37
4f	20	thyroid	65	80	65	52	43			-17
		thyroid						70 ¹	52	63 ¹
5m	11	thyroid		27	25	28	22			-21
		thyroid						26 ¹	31	18 ¹
6m	13	thyroid	74	37	45	46	43	37		-6
7f	11	thyroid	52	34	35	36	37	32		3
8f	23	thyroid		57	50	52	63	64	58	21
9m	16	thyroid		57	45	48	59			23
		thyroid						67 ¹	63	30 ¹
10f	11	thyroid		27	22	28	29			4
11f	13	thyroid		33	22	28	25	29	23	-11
Mean	14		54	41	36	37	39	36		

Positive responses to thyroid extracts										
12f	15	thyroid	92	82	46	50	82	73		64
13f	17	thyroid	41	42	39	47	68	44	38	45
14f	26	thyroid	56	53	61	52	120	84	74	130
15f	19	thyroid		52	41	37	53	86		43
16m	21	thyroid	40	36	50	46	131	78	78	183
17f	32	thyroid	79	59	61	60	83	88	65	38
18f	22	thyroid	55	39	50	50	79	37		58
		thyroid							65 ¹	55 ¹
Mean	22		60	51	50	49	88	70	63	

Responses to non-thyroid tissue extracts										
19m	13	brain	38	39	32	36	43	52	34	20
20f	12	muscle	28	34	42	33	45	36		36
		thyroid ¹							47 ¹	30 ¹
21f	12	liver		16	14	18	29	21		61
22f	12	muscle	34	28	32	34	33	30		-3
23f	15	muscle	87	43	41	49	49	49	43	0
24	14	liver	49	30	38	37	42	Died		13
25f	28	brain	65	50	52	56	56	46		0
Mean	15		50	34	36	37	42	39		
Mean for all groups			55	42	40	41				

¹ Response to second injection not included in means or in figure 2. For this reason the positive response to the second injection in no. 4 is placed among the negative results and the thyroid injection into no. 20 is placed among the non-thyroid extracts.

liver. No animal was placed in the positive group unless its oxygen consumption was at least 40 per cent higher on the day after the injection than on the day before.

This may seem an unnecessarily rigorous test, but it was held essential because of the marked daily variability in oxygen consumption. Possibly one or two animals (nos. 3 and 9) in the negative group might be properly considered to have shown positive responses. *Fish no. 9*, in particular, showed a marked increase in oxygen consumption after the second injection of thyroid extract.

In figure 2 the average oxygen consumption for the 25 white grunts included in table 1 is given on the day of injection and on the four days immediately preceding and including the day of injection. Oxygen consumption was highest at the beginning of the run (55 mm.³ per minute). For many fish this day was the first in the chamber, a time when oxygen consumption is always high. During the next three days the average oxygen consumption was remarkably constant, the actual values being 42, 40 and 41 mm.³ per minute.

TABLE 2. EFFECT OF EXTRACTS GIVING EITHER A POSITIVE RESPONSE IN SOME FISH OR A NEGATIVE RESPONSE IN OTHER FISH

EXTRACT NO.	RESPONSE, ON DAY AFTER INJECTION					
	Negative			Positive		
	Fish No.	% change in O ₂ cons.	Wt.	Fish No.	% change in O ₂ cons.	Wt.
			grams			grams
8	2	21	15	18	58	22
11	20	30	12	4	63	20
	9	23	16	18	76	22
15	5	18	11	12	64	15
	6	-6	13			
	7	3	11			
Mean.....		15	13		65	20

After injection the average oxygen consumption of the seven fish given extracts of non-thyroid tissue showed no change. This response was also found, of course, in the eleven fish of the negative group. In the seven fish of the positive group, however, the oxygen consumption on the day after the injection averaged 88 mm.³ per minute on the first day and 70 mm.³ per minute on the second day after injection. Compared to the mean oxygen consumption of the whole group prior to injection, this represents a substantial increase. Actually the increase was not quite as great as would appear from figure 2, since the mean level for the positive group before injection is somewhat higher than the mean level for the group as a whole. This is due to the fact that the weight of the positive group on the whole is higher than the combined average weight of all groups. However, the difference in no way alters the significance of the results.

Out of the 13 extracts prepared, 5 gave only positive responses, 5 gave only negative responses, and 3 gave positive responses in some fish and negative responses in others. The responses to the last group of extracts are shown in table 2. It is seen that all fish giving positive responses weighed 15 grams or more and that all those giving negative responses weighed 16 grams or less. These facts strongly suggest that the character of the response is determined in part by the weight of the fish. This supposition is borne out by the data of table 1, where it is seen that all animals

except two (*nos. 4 and 9*) giving a negative response weigh 15 grams or less and that the average weight for the group is 14 grams. On the other hand, all of the animals in the positive group weigh 15 grams or more and the average weight for the group is 22 grams.

DISCUSSION

The failure of some animals to respond to parrot fish thyroid extract may have two possible explanations. Either the fish used were unresponsive or the injected extracts were inactive. In these experiments both possibilities seem implicated. As we have already seen, fish below a certain weight will not respond. Whether there are limiting factors other than weight is not known. The data of table 1 suggest that sex differences may also be involved since six of the seven fish showing a positive response were females. However, we do not feel that our data are sufficient to justify such a conclusion, especially when it is noted that the one male fish (*no. 16*) in the positive group gave the greatest response. Nor can any differentiation between the negative and positive groups be made on the grounds of sexual maturity since all animals were proved to be sexually active.

Unquestionably some of our negative results were due to the use of inactive extracts. Our crude method of preparation was probably not conducive to the preservation of the metabolic stimulating factor. However, not all of our negative results can be explained as due to the use of inactive extracts, as is clearly shown in table 2.

It is fortunate that the negative group includes two animals (*nos. 4 and 8*) which weigh 20 grams or more, for they demonstrate that large animals injected with inactive thyroid extracts do not show an increase in oxygen consumption detectable 24 hours after injection resulting from the handling and excitement. In the group injected with non-thyroid tissue extracts, only one fish (*no. 25*) can be classed as a large animal. Undoubtedly more control experiments, on animals weighing 20 grams or more and injected with inactive thyroid extracts or non-thyroid extracts, would be desirable. At the time the present work was done, the importance of size was not appreciated.

In table 1, one of the fish (*no. 21*) injected with liver extract gave what must be termed a positive response. From other experiments we suspected that liver extracts were toxic and possibly this fact accounted for the prolonged excitation following injection. With this one exception, no tissue extract gave a positive response. Thus we feel that the evidence at hand definitely indicates extracts of the parrot fish thyroid have a special capacity to increase oxygen consumption in white grunts.

There seems to be no *a priori* reason why smaller animals should be less sensitive to thyroid extract than larger animals. The oxygen consumption of the small animals did not deviate from the average set by the larger fish. The small fish (weighing less than 15 grams) had an average oxygen consumption of 2.46 mm.³ per gram per minute. Comparing this with the average oxygen consumption (2.37 mm.³ per gram per minute) of the larger group, it is evident that there is no significant difference between the two groups. Thus, there is no indication of any metabolic differences between large and small grunts which would account for their different sensitivities to

thyroid extract. In both groups data were collected after the fish were adapted to the respiration chambers and after their oxygen consumption rates had stabilized. No attempt was made to study the effect of thyroid extracts on the oxygen consumption of animals which were stimulated to greater activity than was the case in these experiments. Spoor (5) and Fry and Hart (9) have developed methods for determining the effect of activity on fish metabolism. According to Spoor (5), in an active goldfish the oxygen consumption at 23°C. to 25°C. may be four times that found in the basal state. Fry and Hart (9) have shown that at 22°C. a swimming goldfish consumes about twice as much oxygen as does one remaining quiet.

Whether the increase in oxygen consumption observed on the injection of thyroid extracts into fish has physiological significance cannot be decided on the evidence presented here. Any assumption based on data from mammalian experiments would be misleading. It must not be forgotten that fish are poikilotherms and are not under the necessity of regulating body temperature as is the mammal. Not until we know more about the relation in fish between bodily activity, temperature, thyroid activity and oxygen consumption will we be able to assess the significance of any metabolic stimulating effect of thyroid extracts.

The fact that thyroid extracts of mammalian origin do not increase oxygen consumption in the fish, while those of teleost origin do, suggests a possible difference between these two compounds. However, it does not necessarily follow that these two extracts have identical chemical composition.

SUMMARY

Extracts were prepared from the thyroid glands of Bermuda parrot fish. These extracts, when injected into white grunts weighing 15 grams or more, produced a significant rise in oxygen consumption within 24 hours after injection. Fish weighing less than 16 grams did not react by increasing their oxygen consumption.

All of our experiments were performed at the Bermuda Biological Station for Research where through the kind offices of Dr. Dugald E. S. Brown, the Director, we were adequately supplied with space and materials. We are also deeply indebted to Mr. L. S. Mowbray of the Bermuda Government Aquarium, whose assistance was invaluable in securing a supply of parrot fish. The work was supported by a grant from the Williams 1900 Fund and by the Bermuda Biological Station for Research, from funds supplied by the American Philosophical Society.

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CONCENTRATION OF RADIOACTIVE IODINE BY THE THYROID GLAND OF THE PARROT FISH, SPARISOMA SP.¹

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ATTEMPTS to determine the rôle played by the thyroid gland in the activities of teleost fishes have been handicapped by the diffuse nature of the gland in these forms. The number of experiments is small, the results in some cases conflicting. Our present knowledge concerning the gland can be quickly summarized.

Anatomically it consists of follicles scattered in the connective tissue along the ventral aorta and afferent branchial arteries (1, 2), except in the swordfish, *Xiphias*, where it is compact (3). The glands of the haddock (4) and of the flounder (5) contain iodine, though in rather small quantities. Thyroid material from several species is reported to accelerate metamorphosis in frog tadpoles (5, 6), though in neither case is a description given of how the thyroid was removed and identified as such. The effect of teleost thyroid extracts on growth rate and body form of fishes has not been reported, but both may be altered by mammalian preparations (7-9). The administration of thyroxine, thyroid tablets or thyroid powder has no apparent effect on oxygen consumption in teleosts (10-12), nor do teleosts treated with thiourea show any reduction in oxygen metabolism (13). It has been found, however, that treatment with thiourea may result in retardation of growth and failure in development of secondary sexual characteristics (14). Furthermore, on the basis of histological changes appearing in the thyroids of the eel and certain flat fishes at metamorphosis, it has been suggested that the gland in these animals is concerned with the pronounced changes that occur in these forms (15, 16). Finally, it has been reported that the teleost thyroid can be activated by pituitary extracts of several groups of vertebrates, including fishes (17, 18).

These fragments of information concerning the teleost thyroid help very little in understanding precisely what rôle the gland plays in the economy of the individual. Recently a discrete, compact thyroid gland has been reported in the Bermuda parrot fish (19). Since these animals are abundant in Bermuda waters they afford good material for studying the functions of the thyroid in teleost fishes. One of the first problems investigated was concerned with the ability of the gland to concentrate iodine, a property which has been well established for the thyroids of higher vertebrates. To examine this the following experiment was carried out.

Small specimens of *Sparisoma*, the Bermuda 'mudbelly,' were injected intra-

Received for publication April 16, 1948.

¹ This work was aided by grants from the Williams 1900 Fund and the American Philosophical Society. It was carried out at the Bermuda Biological Station for Research. We are indebted to Dr. Dugald E. S. Brown, Director of the Station, for many helpful suggestions, and to Mr. L. S. Mowbray, Director of the Government Aquarium, for identification of our animals and for permission to use fish from the Government pool.

peritoneally with radioactive iodine.² The only method available for detection of the radioactive substance in the tissues was the autographic technique which has been described by a number of workers. A recent excellent review is that of Axelrod (20). I¹³¹ is known to produce good autographs of tissues which contain large enough concentrations of it, presumably because of its beta emissions. It has a beta radiation of 0.6 MEV and gamma radiations of 0.357 and 0.080 MEV. "No radioautographic data have been reported in the literature for a pure gamma emitter. The range of gamma radiation is great and limited ionization occurs; therefore, 10 to 100 times the radioactivity would probably be required to obtain an autograph from a gamma emitter as compared to an alpha or beta emitter" (Axelrod, 20). Beta emitters, however, give good autographs of materials containing them in sufficient quantity. It is of interest also to note that beta rays with lower energy values, such as those from I¹³¹, give autographs of better resolution than those with higher ones, such as P³², with beta energy of 1.72 MEV (20).

When measured at Stamford on April 29th the 10-cc. sample used in these experiments contained 65-70 microcuries of I¹³¹. This material has a half-life of 8.0 days. Since some delay was experienced in shipment, when received in Bermuda, May 7th, it contained approximately 3.3 microcuries per cc. Nothing was known of the ability of fish thyroids to concentrate iodine. Since only the one sample was available we varied the dosage widely but tried to make sure that at least some of the animals would receive enough material to affect X-ray film. Accordingly eight animals about 20 cm. in length were injected with doses varying from 0.8 to 2.5 cc. Each animal thus received between 2.6 and 8.3 microcuries. The animals were killed at intervals of 6, 12, 24 and 48 hours after injection. From each fish the thyroid, auricle and a piece of gill were removed for study. The auricle was used as an example of nonthyroid tissue whose ability to concentrate iodine might be compared with that of the thyroid. The gill was examined because in most teleosts thyroid follicles are scattered in the region of the gills and we were curious to see if this might be true also in these forms, even though they do possess a fairly compact gland. The tissues were placed on a glass slide and dried in an oven at 50°C. Such drying of material preparatory to making autographs of it concentrates any radioactive material it may contain into a smaller volume and also flattens the tissues so that autographs of better resolution are produced. After 12 hours in the oven the slide was removed and applied to a piece of dental x-ray film which was left in its film cover. The penetrating power of beta radiations is small. A preliminary trial showed that our material would not affect the film through both the cover and the glass slide, so in all experiments the slide was mounted on the film with the tissues in contact with the film cover. The film and slide were held together firmly by adhesive tape and were left in contact for periods ranging from 56 hours to 8 days.

When the films were developed every case showed autographs of both thyroid and gill region (figs. 1, 2). Although the gill concentrated the material less than did the thyroid, with long exposures this difference was masked (fig. 4), but in shorter

² The iodine was prepared in Columbia University's cyclotron and separated from contaminating materials in the research laboratories of the American Cyanamid Company at Stamford, Connecticut. We are greatly indebted to Drs. R. O. Roblin and D. J. Sally of this laboratory for securing, purifying and measuring the potency of this material, and for shipping it to us in Bermuda.

ones the autographs of the thyroid and of the gill were obviously different in intensity (fig. 3). The portion of the film under the auricle was not affected (figs. 1, 2).

It was thus clear that both the thyroid and the gill tissues had concentrated I^{131} in sufficient degree to affect the x-ray emulsion. Although the iodine must have been widely distributed to the tissues of the body, its concentration in the auricle, and presumptively in other nonthyroid tissues as well, was too small to affect the film. Why the tissues in the region of the gill also concentrated the radioactive iodine has not been determined. The most obvious explanation is that here, as in other teleosts, some thyroid follicles are to be found in the neighborhood of the gill. Histo-

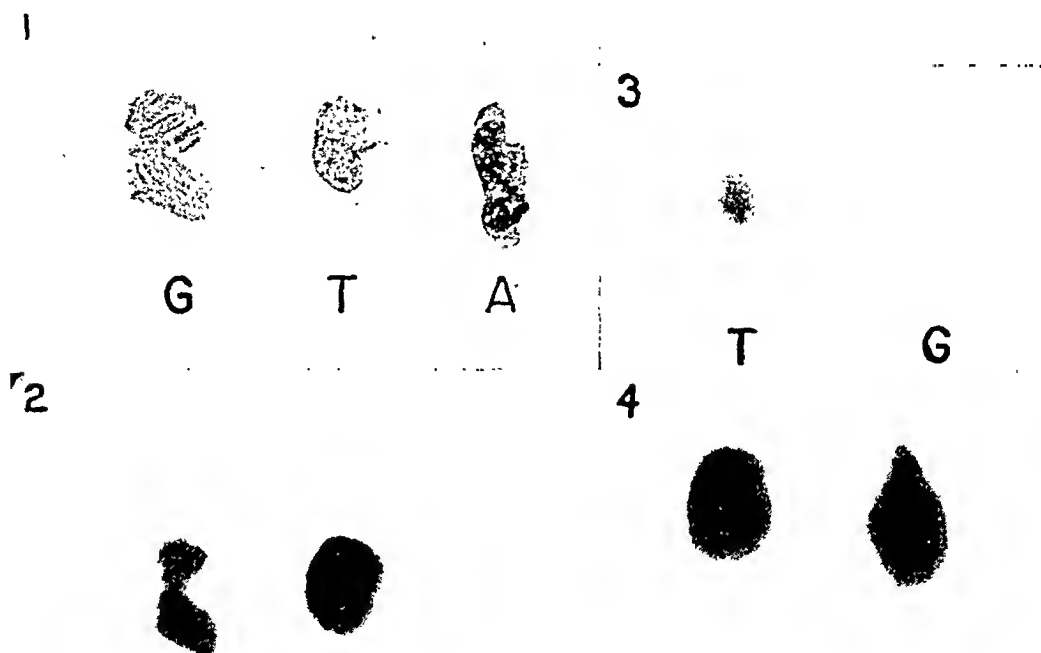


Fig. 1. PHOTOGRAPH OF DRIED TISSUES of parrot fish

Fig. 2. RADIOAUTOGRAPH OF TISSUES shown in figure 1

Fig. 3. RADIOAUTOGRAPH OF PARROT FISH TISSUES, exposure time, 56 hours

Fig. 4. SAME AS FIGURE 3, exposure time $6\frac{1}{2}$ days. A, auricle; G, piece of gill tissue; T, thyroid

logical study of this region to determine whether or not this is the case has not yet been made.

DISCUSSION

The methods that can be used to study the activity of the diffuse thyroid gland present in most teleosts consist chiefly of measuring cell height and computing follicle size and number. These are tedious and yield results of questionable accuracy. Efforts to determine the effects of hypophysectomy on the thyroid of *Fundulus*, for example, have produced no results that could be interpreted (unpublished data). The compact gland of the parrot fishes can be studied by more precise and convenient methods. Since the gland concentrates radioactive iodine, quantitative methods may be employed to measure the concentration of this material by the gland under

different experimental conditions. Variations in activity coincident with seasonal changes, with different phases of growth and the sex cycle, and the relation of the thyroid to the pituitary gland, can all be more effectively studied by this means. The results of such experiments should help a great deal in understanding the rôle played by the thyroid in the bony fishes in particular and to some extent in the cold-blooded forms in general. More than this, it seems reasonable to suppose that adequate knowledge of the functions of the thyroid in such forms will illuminate to some degree at least the activities of the gland in warm-blooded vertebrates as well. In birds and mammals the thyroid is involved in the processes which maintain a high metabolic rate and constant body temperature. The concern of the thyroid in these processes must have developed in connection with, or at least was greatly exaggerated by, the evolution of the homoiothermal condition. The little evidence we have indicates that in fishes, at any rate, the thyroid is but little concerned in metabolic regulation. Yet cyclic changes in the gland of several fishes indicate that it plays some rôle in the activities of the individual, a rôle presumably more primitive than that in warm-blooded forms. When its functions in cold-blood forms are known they might well provide a better understanding of similar functions in warm-blooded animals which are masked by the dominant activities associated with metabolic regulation.

SUMMARY

The thyroid gland of the parrot fish, *Sparisoma*, unlike that of most teleosts, is a compact one. Like the thyroids of higher vertebrates it concentrates radioactive iodine. The extent to which the gland concentrates this material under different seasonal and experimental conditions can now be studied by the quantitative methods available for measuring such radioactive isotopes.

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POTENTIATION OF THE HYPERTENSIVE EFFECTS OF DESOXYCORTICOSTERONE ACETATE (DCA) BY VARIOUS SODIUM SALTS¹

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THE rise in blood pressure in essential hypertension has long been correlated with changes in salt metabolism. While moderate restriction of salt has become incorporated into the routine therapy of this disease in many centers, Grollman (1) confirmed by others (2) has recently reported a beneficial effect with rigid restriction of sodium in some patients. In animal experiments, Grollman and Harrison (3) observed an increase in the survival time of renal hypertensive rats fed a diet low in sodium, but Grollman (4) found no decrease in pressure in renal hypertensive dogs. Manwaring (5) obtained a lowering of the blood pressure in renal hypertensive dogs on a low chloride diet.

On the basis of his experimental findings, Selye (6) has suggested that the cause of essential hypertension might reside in an overproduction of adrenal cortical hormones. This would provide some explanation for the occasional beneficial effect of salt restriction observed clinically.

Investigating this further we have found that a hypertensive syndrome can be elicited in the rat by small doses of desoxycorticosterone acetate (DCA) without the use of other intensifying measures (7). The doses used did not exceed 300 γ /day. The substitution of 1 per cent saline for the animal's drinking water aggravated the condition without altering it qualitatively. Changes in renal function occurred which were analogous to those which have been observed in essential hypertension in man, and there was also an elevation in plasma sodium, accompanied by a decrease in plasma potassium and chloride, with a consequently progressively increasing Na/Cl ratio.

In view of our failure to elicit any comparable change in electrolytes in rats made hypertensive by renal compression (8), as well as the clinical observation that salt restriction is beneficial and an electrolyte alteration demonstrable only in some cases we have, for the present, considered that the adrenal cortex may play a primary rôle only in some cases of essential hypertension. Implicit in this is the suggestion that such cases be classed as instances of 'hormonal' rather than 'essential' hypertension.

Because of its practical features, the mechanism of action of DCA in this process requires further elucidation. Perera and Blood (9) after administering DCA to normal and hypertensive subjects for one week noted an elevation in systolic and diastolic pressures only in the latter group. Since changes in salt and water metab-

Received for publication May 3, 1948.

¹ This work was supported by a grant from the Life Insurance Medical Research Fund.

² This work was done during the tenure of a Life Insurance Medical Research Fellowship.

olism were the same in both groups they concluded that the effects of DCA on blood pressure and on salt metabolism were probably independent processes. In another series (10) they reported an abnormal salt metabolism in hypertensive patients which they considered might be referable to altered renal function. Knowlton *et al.* (11) have studied the effect of saline feeding in rats under treatment with DCA and concluded that the potentiation of the effects observed was specifically due to the sodium ion, since potassium chloride was without effect. While it is true that the absence of a potentiating effect in the case of potassium chloride was probably because no added sodium was present, it might also have been referable to a beneficial effect of added potassium. It is not possible to distinguish between the three variables of potassium, sodium and chloride by loading the organism with only two salts such as NaCl and KCl.

It appeared that some clarification of this problem might be obtained if the animal were treated with a series of salts having, if possible, the same single variable in each.

EXPERIMENT I

Initially, sodium salts of normal organic metabolites were used. These were chosen with the idea that the organic part of the molecule would probably break down without influencing the electrolyte pattern itself. In the first experiment, sodium glycerophosphate and sodium glutamate were tested. (Preliminary work in which normal animals received these salts in their drinking water for five weeks showed them to be nontoxic.) In both instances, it might be expected that the organism would be faced with the need for handling an excess of sodium.

Four groups of 20 male albino rats were maintained for 25 days. Throughout the experimental period they were fed Purina Fox Chow *ad lib.* Half of each group was used for renal function studies (12), the other half for plasma electrolyte determinations (13). Blood pressure, using the method of Byrom and Wilson (14) with ether, was determined in all animals. All investigations were carried out during the third week of the experiment, and when completed the animals were killed and the hearts and kidneys weighed after fixation. Later, the microscopic appearance of kidney sections was studied.

Group 1 served as intact control and received tap water *ad lib.* The animals of *group 2* received a subcutaneous implant of DCA (one third of a 75 mgm. Schering Cortate pellet) on the first and tenth days of the experiment, and 1 per cent saline as drinking water. *Group 3* received a similar administration of DCA and 2.9 per cent sodium glutamate as drinking water, while *group 4* received DCA with 2.7 per cent sodium glycerophosphate. Each of these waters contained equivalent amounts of sodium. They were fed *ad lib.*, and in all cases were liberally ingested by the animals. Although water intake was not recorded, the NaCl water bottles were those most often refilled. Table 1 presents all the pertinent data.

Blood pressure. DCA raised the blood pressure, as substantiated by the increase in heart weight observed in all groups receiving the steroid. While the average pressure in the groups fed glutamate or glycerophosphate was lower than that of the saline-fed group (2), no real comparison is possible because some animals, particularly in *group 4*, were moribund when the pressure was recorded. For this reason,

the range of blood pressure is given in the table rather than the standard deviation. Certainly, however, the effect on blood pressure was not greater in the groups fed glutamate or glycerophosphate than in the saline-fed group and seems to have been less.

TABLE 1

GROUP NO.....	1	2	3	4
Treatment.....	Control	DCA-Saline	DCA-Glutamate	DCA-Glycero-phosphate
Blood pressure, mm. Hg	98 (80-112)	130 (108-170)	116 (90-140)	117.8 (80-170)
C_{IN} , cc/100 cm ²	0.32 ±0.02	0.25 ±0.08	0.28 ±0.08	0.15 ±0.06
C_{PAH} , cc/100 cm ²	2.35 ±0.29	1.95 ±0.15	1.77 ±0.42	0.80 ±0.30
Tm_{PAH} , mgm/100 cm ²	0.129 ±0.010	0.117 ±0.015	0.093 ±0.017	0.038 ±0.012
FF as %	14.0 ±1.4	12.3 ±3.7	15.4 ±3.0	20.3 ±7.7
C_{PAH}/Tm_{PAH}	18.4 ±2.3	17.3 ±1.1	18.3 ±1.9	21.2 ±6.6
mEq. Na	151.5 ±2.0	150.0 ±1.7	148.6 ±2.4	149.5 ±2.6
mEq. Cl	102.0 ±2.0	95.4 ±2.4	90.1 ±6.3	90.8 ±4.9
mEq. K	5.7 ±0.3	4.7 ±0.4	3.2 ±0.6	3.7 ±0.8
Heart, wt., mgm/100 cm ²	169 ±13	203 ±26	209 ±32	211 ±51
Kidney wt., mgm/100 cm ²	458 (437-492)	591 (521-677)	632 (551-805)	1150 (980-1310)
Initial wt., gms.	58	60	62	62
Final wt., gms.	157	131	136	109
Animals with kidney lesions	0/18	6/16	4/13	13/13

Renal function. As we have previously observed (7), at this time (three weeks) renal function was impaired in the DCA-saline group, with reductions of slight degree in the glomerular filtration rate (GFR, C_{IN}), renal plasma flow (RPF, C_{PAH}) and functional tubular excretory mass (Tm_{PAH}), while no renal ischemia, measured as C_{PAH}/Tm_{PAH} , was evident.

These changes were also present in the DCA-glutamate group (3), with evidence of a greater loss of tubular function as shown by a greater decrease in Tm_{PAH} . Renal plasma flow was also considerably reduced in this group, but in proportion to the loss of tubular excretory function, so that again no ischemia was present, C_{PAH}/Tm_{PAH} remaining within normal limits. Glomerular function, C_{IN} , was relatively well preserved.

In group 4, which received DCA with glycerophosphate, severe renal damage

occurred. The glomerular filtration rate was halved, while renal plasma flow and tubular excretory mass were reduced to about one third of normal. Although badly damaged, the filtration mechanism was preserved better than the tubular, with consequent elevation of the filtration fraction (FF). Notwithstanding the widespread damage, the plasma flow per unit of residual tubular excretory tissue, C_{PAH}/Tm_{PAH} , was within normal limits.

Plasma electrolytes. It is clear that DCA-saline treatment for three weeks produced no change in plasma Na, while plasma K and Cl were depressed. This agrees with our previous work, where a decreased plasma K and Cl preceded the appearance of an elevation in the plasma Na. Similarly, plasma Na was unchanged in the groups receiving glutamate and glycerophosphate, while potassium and chloride were depressed further than in the DCA-saline group.

Histology and organ weights. How poorly renal function was actually maintained is brought out by the significant increase in kidney weight which occurred in the group receiving DCA with saline, the marked enlargement in the group receiving DCA with glutamate and the tremendous enlargement seen in the DCA-glycerophosphate group.

Histologically, minimal lesions suggesting early glomerular sclerosis were observed in both the DCA-saline and DCA-glutamate groups, while in the DCA-glycerophosphate group renal structure was completely destroyed in many areas. There was widespread diffuse tubular atrophy and necrosis with scarring, so that whole masses of tubules were replaced with scar. Those glomeruli which were preserved were shrunken, with consequent enlargement of the capsular space and even such glomeruli were few. No vascular lesions were apparent.

DISCUSSION. The administration of DCA with the sodium salt of certain organic compounds appears to be more damaging to the kidney than when DCA is given with NaCl. This is shown by the greater functional derangement and the greater compensatory increase in renal mass as well as by histological examination and electrolyte study. This increased damage is not accompanied by a greater increase in blood pressure.

Several conclusions may be drawn from these facts. It appears that the degree of elevation in blood pressure is not dependent on the degree of derangement of renal function, nor on the degree of interference with the electrolyte pattern as studied here. Electrolyte change seems to follow renal damage rather than blood pressure increase. Since an elevation of the blood pressure and an aggravation of renal functional derangement both occur following the ingestion of sodium in the three different salts studied, this factor would seem to be of etiological significance in both processes.

Special mention must be made of sodium glycerophosphate. So dramatic were the changes in renal function in the group receiving this compound that it seems likely it exerts a renal damaging influence beyond that of its sodium component.

EXPERIMENT 2

A second experiment was now undertaken along the lines of the first but using different sodium salts to rule out the possibility that the previous choices had been fortuitous. The salts employed were sodium citrate and sodium succinate.

Six groups of 10 male albino rats were maintained for 18 days on a diet of Purina Fox Chow *ad lib*. This experiment was maintained for one week less than the preceding, because the DCA-glycerophosphate animals in that study had been so badly damaged at the end of three weeks. Renal function studies were performed on the 15th and 17th days of the experiment, in each case preceded by the determination of blood pressure. The animals were killed on the 18th day and the kidneys and hearts were weighed. Electrolytes were not studied in this experiment.

Group 1 served as intact control and received tap water *ad lib*; *group 2* received 2.33 per cent sodium succinate as drinking water; *group 3* drank 1.69 per cent sodium citrate; *group 4* received a DCA implant subcutaneously (one third of a 75 mgm.

TABLE 2

GROUP NO.....	1	2	3	4	5	6
Treatment.....	Control	Succinate	Citrate	DCA-Saline	DCA-Succinate	DCA-Citrate
Blood pressure, mm. Hg	99 (80-110)	96 (84-108)	98 (88-118)	124 (110-140)	110 (100-140)	106 (88-140)
No. with B.P. above 120	0/8	0/8	0/9	7/10	1/10	2/9
C _{IN} , cc/100 cm ²	0.34 ±0.06	0.38 ±0.05	0.35 ±0.12	0.30 ±0.08	0.26 ±0.08	0.25 ±0.09
C _{PAH} , cc/100 cm ²	2.22 ±0.48	2.17 ±0.33	2.19 ±0.34	2.06 ±0.34	1.95 ±0.71	2.04 ±0.30
Tm _{PAH} , mgm/100 cm ²	0.124 ±0.011	0.124 ±0.011	0.123 ±0.010	0.130 ±0.017	0.111 ±0.019	0.107 ±0.021
FF as %	15.3	17.5	15.9	14.5	13.4	12.2
C _{PAH} /Tm _{PAH}	17.9	17.5	17.9	15.8	17.5	19.0
Kidney wt., mgm/100 cm ²	479 ±43	485 ±35	483 ±23	528 ±30	561 ±31	660 ±83
Heart wt., mgm/100 cm ²	183	169	167	175	173	187
Initial body wt., gms.	71	67	66	79	82	85
Final body wt., gms.	140	130	139	137	131	131

Schering Cortate pellet) on the first and tenth days of the experiment and 1 per cent saline as drinking water; *group 5* also received DCA pellet implants but drank 2.33 per cent sodium succinate; and *group 6* consisted of animals with DCA pellet implants drinking 1.69 per cent sodium citrate. Each of these drinking waters contained equivalent amounts of sodium and were in all cases liberally ingested by the animals. The NaCl water bottles were again those most often refilled. Table 2 presents the pertinent findings.

Blood pressure. No change in blood pressure was observed in the three groups not receiving DCA. In clear-cut contrast, an elevation in the average blood pressure occurred in all three groups receiving DCA, significant, however, only in the DCA-saline group. Taking a pressure of 120 mm. Hg as representing the highest normal figure observed in the 25 determinations on *groups 1-3*, it is readily appreciated that most of the determinations in the DCA-saline group lay above this figure, while in the DCA-succinate group, one animal in ten, and in the DCA-citrate group, two in nine exceeded this arbitrary figure. Regardless of the absolute validity of the actual

figures obtained it is clear that the pressure-raising effect of DCA was greatest in the DCA-saline group. (It has previously been shown that saline alone is quite ineffective in this respect.) That no increase in heart weight occurred in any group in this experiment is quite understandable in view of its brief duration although an increase at this time did appear in the next experiment.

Renal function. The administration of either sodium succinate or sodium citrate alone was entirely without effect on any renal function. In contrast, renal function was slightly but consistently depressed in the three DCA-treated groups, with the least change in the over-all picture in the DCA-saline group. In this latter group function was essentially normal, and the depression of C_{IN} and C_{PAH} , although interesting since it follows the same trend shown by groups 5 and 6, was not statistically significant. This confirms our previous observations at two weeks with DCA-saline. In the DCA-succinate and DCA-citrate groups, C_{IN} , C_{PAH} and Tm_{PAH} were all somewhat depressed. C_{PAH}/Tm_{PAH} remained within normal limits in all groups indicating that there was no renal ischemia.

Histology and organ weights. Histological examination of the kidneys revealed no pathological changes in any of the control groups. In the groups receiving DCA there were suggestions of early glomerular sclerosis and slight tubular atrophy in the kidneys of some animals. These minimal lesions were not encountered to any greater degree in any one group.

The indication of a more severe renal functional involvement in groups 5 and 6 was further substantiated by the fact that while kidney weight per 100 gm² was significantly greater than normal in the DCA-saline group, it was considerably greater in the DCA-succinate and DCA-citrate groups.

DISCUSSION. This substantiates the first experiment. Apparently excess ingestion of the sodium ion by animals under treatment with DCA intensifies the renal effect of this steroid. There is strong evidence that an intensification of renal damage is not necessarily reflected by a proportionate increase in the hypertension.

It is likewise apparent that the excessive renal damage observed in animals receiving DCA with sodium glycerophosphate, in the first experiment, was not due to its sodium content alone for this did not occur with sodium glutamate, citrate or succinate, all of which were more damaging than saline. The possibility suggests itself rather strongly that glycerophosphate was specifically damaging because it furnished excess phosphate. A third experiment in this series was now undertaken.

EXPERIMENT 3

Eight groups of 10 male albino rats were maintained for 15 days on a diet of Purina Fox Chow *ad lib*. Renal function studies were performed on the fifteenth day of the experiment, preceded by the determination of blood pressure on the fourteenth. The animals were killed immediately after the clearance study and the kidneys and hearts fixed and weighed.

Group 1 served as intact control and received tap water *ad lib*; group 2 received 2.78 per cent sodium sulfate as drinking water; group 3 received 3.1 per cent dibasic sodium phosphate and group 4, 2.4 per cent monobasic sodium phosphate. Group 5 received a DCA pellet implant (one third of a 75 mgm. Schering Cortate pellet) on the first day of the experiment, and 1 per cent saline as drinking water; group 6

consisted of animals with DCA pellet implants drinking 2.78 per cent sodium sulfate, *group 7* of animals with DCA pellet implants drinking 3.1 per cent dibasic sodium phosphate and *group 8* of animals with DCA implants drinking 2.4 per cent monobasic sodium phosphate. Each of these drinking waters contained the same concentration of sodium and were in all cases liberally ingested by the animals. The NaCl water bottles were those most often refilled. Table 3 presents the pertinent findings.

TABLE 3

GROUP NO.....	1	2	3	4	5	6	7	8
Treatment.....	Control	Na ₂ SO ₄	Na ₂ HPO ₄	NaH ₂ PO ₄	DCA-NaCl	DCA-Na ₂ SO ₄	DCA-Na ₂ HPO ₄	DCA-NaH ₂ PO ₄
Blood pressure, mm. Hg	92 ±8	88 ±6	86 ±11	93 ±8	113 ±11	110 ±11	116 ±16	100 ±32
No. with blood pressure > 110	0/9	0/9	1/7	0/8	4/9	2/6	5/8	2/6
C _{IN} , cc/100 cm ²	0.40 ±0.09	0.47 ±0.14	0.37 ±0.09	0.31 ±0.12	0.30 ±0.12	0.36 ±0.16	0.28 ±0.13	0.22 ±0.09
C _{PAH} , cc/100 cm ²	2.78 ±0.40	2.53 ±0.54	2.72 ±0.63	2.55 ±0.75	2.52 ±0.48	2.57 ±0.47	2.09 ±1.42	0.80 ±0.45
Tm _{PAH} , mgm/100 cm ²	0.145 ±0.017	0.125 ±0.022	0.133 ±0.018	0.120 ±0.033	0.119 ±0.021	0.123 ±0.016	0.105 ±0.035	0.050 ±0.031
FF as %	14.4	18.5	13.6	12.1	12.0	14.0	13.4	27.0
C _{PAH} /Tm _{PAH}	19.1	20.2	20.4	21.2	21.1	20.8	19.9	16.0
Kidney wt., mgm/100 cm ²	453 ±18	479 ±29	486 ±49	584 ±90	596 ±62	581 ±71	852 ±190	1135 ±94
Heart wt., mgm/100 cm ²	190 ±12	193 ±16	202 ±17	213 ±27	227 ±18	229 ±13	244 ±6	190 ±21
Initial wt., gms.	59	64	70	79	79	87	83	71
Final wt., gms.	132	131	135	145	143	150	161	85
24-hour intake per 100 grams in cc.					46.3	31.3	29.3	25.4

During the course of this experiment an outbreak of pneumonia occurred in the colony of origin. The animals in this experiment received prophylactic injections of penicillin which may account for the increase in the control values for C_{IN} and C_{PAH} here as compared with our usual findings.

Blood pressure. No change in blood pressure was observed in the four groups not receiving DCA, although one frankly hypertensive animal was observed in *group 3* drinking dibasic sodium phosphate. Again, in clear-cut contrast, elevations of pressure occurred in all four groups receiving DCA. This was true even in the case of *group 8*, DCA with monobasic sodium phosphate, where although only 6 animals in the group survived what was apparently an intensely damaging process and were in relatively poor condition, still two of the six exhibited a marked elevation in pressure. With the exception of this group the degree of elevation in all DCA groups was about the same. In support of these findings, heart weight was significantly increased in all groups receiving DCA except in *group 8* where the animals were greatly debilitated. It is not possible to explain at this point why, in the previous experiment, cardiac hypertrophy did not develop within a similar period.

Renal function. While the administration of the sodium salts without DCA was without significant effect on renal function, this statement is only partially true in respect to phosphate. Thus, in *group 4*, receiving monobasic sodium phosphate, there was a trend toward impairment, which was borne out by the increase in renal weight observed at autopsy. Function proportionate to renal mass was obviously not maintained, indicating the damaging effect of phosphate alone. It should be emphasized that since these drinking waters were matched for sodium content, *groups 4* and *8* received almost double the amount of phosphate given *groups 3* and *7*.

No statistically significant change in renal function was exhibited by either *group 5* or *6*, DCA with saline and sodium sulfate drinking waters, respectively, although the increase in kidney size indicates that function was maintained only by hypertrophy. In contrast, function was impaired in both *groups 7* and *8*, DCA with dibasic and monobasic sodium phosphate, respectively. In *group 7*, receiving less phosphate, the maintenance of renal function even at values somewhat below the control level involved a great increase in renal size. In *group 8*, the changes in 15 days here were comparable with those previously observed in animals given DCA with sodium glycerophosphate for 25 days. In this group, renal plasma flow and functional tubular mass were greatly reduced, while the filtration rate was reduced to a lesser extent. The parallel between the amount of phosphate ingested and the degree of renal damage is clearly indicated by the comparative renal weights in *groups 7* and *8*.

The average water intake of each DCA group for a period of 24 hours is shown in table 3.

Histology. Essentially no histological change was observed in the kidneys of those animals which received salts alone, with the exception of *group 4* (drinking monobasic sodium phosphate) where hypertrophy of the tubules, enlargement of the glomeruli and hemorrhage into the capsular space was seen in some cases. In the groups receiving DCA, only minimal glomerular sclerosing changes were occasionally observed where this treatment was coupled with NaCl or Na₂SO₄. In marked contrast, frank renal pathology was observed in the groups receiving DCA with sodium phosphate. These changes were less marked in the group receiving the dibasic salt and more extreme in the group receiving the monobasic. They were the same in character as those previously observed following the administration of DCA with sodium glycerophosphate.

DISCUSSION. This experiment demonstrates that phosphate is particularly noxious when given to a DCA-treated animal and may even damage the intact kidney. This clarifies the situation previously observed with glycerophosphate.

COMMENT

In these experiments in which DCA was given with various sodium salts, it was noted that the intensification of effects was dependent on the excess ingestion of the sodium ion itself. This confirms the findings of Knowlton *et al.* (11). In the course of the investigation an intensely damaging effect occurred when DCA was given with sodium glycerophosphate. This was traced to the phosphate ion which was excessively damaging in the presence of DCA and could even damage the kidney of the otherwise intact animal when given as monobasic sodium phosphate. Hence, while

sodium specifically aggravates the disease process produced by DCA, it is clear that other ions may be equally important.

It appears that the changes in electrolyte pattern which follow the administration of DCA parallel the interference with renal function. This supports the conclusions of Perera and Blood (10) and of Farnsworth (15) that the electrolyte change is dependent on interference with normal renal function.

Finally, it was also possible to make further observations on the correlation between abnormal renal function and hypertension. While it has been noted consistently that the rise in pressure antedates any significant change in renal function, and that the hypertension is not aggravated by increased renal damage, the conclusion that DCA hypertension is entirely independent of the kidney is not yet warranted. In every instance in which the kidneys were weighed it was noted that the maintenance of renal function within normal limits involved a compensatory hypertrophy of the kidney, suggesting that DCA must cause some initial intrarenal derangement. Whether this derangement causes the hypertension or whether these two effects are separate cannot yet be answered with certainty.

SUMMARY

The action of sodium chloride in intensifying the effects of DCA on renal function, blood pressure and electrolyte pattern seems specifically referable to the sodium content. Phosphate may be excessively damaging to a kidney under the influence of DCA. The derangement in plasma electrolyte pattern caused by DCA appears to parallel the kidney damage. The intensification of the renal damage caused by DCA and salts is not necessarily accompanied by an intensification of the hypertension. While the increase in blood pressure following the administration of DCA antedates significant change in renal function, the presence of renal hypertrophy indicates that some renal change has occurred.

The authors wish to thank Dr. E. Schwenk, and Mr. L. Casey of the Schering Corporation Ltd., for the Cortate pellets and Dr. W. P. Boger of Sharp and Dohme Inc., Glenolden, Pa., for the sodium p-aminohippurate used in this work.

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ACID AND ALKALINE PHOSPHATASE LEVELS IN CONSECUTIVE SEMEN EJACULATES FROM BULLS¹

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STERILITY in males and spermatozoa physiology have been studied with increased interest during recent years. The possible rôle of enzyme systems in the provision of spermatozoan energy has been the object of a number of investigations (1). Extensive reports by the Gutmans and their associates have demonstrated a high concentration of a specific acid phosphatase in the prostatic tissue of the human being (2) and of the Rhesus monkey (3). Further study showed that the enzyme appears in the prostate gland after puberty (4) and precociously in that of prepubertal Rhesus monkeys following testosterone propionate treatment (3). Very little acid phosphatase activity was found in the prostate gland of the sexually mature dog, cat, rabbit, guinea pig and rat; that of the rat, however, contained considerable quantities of alkaline phosphatase (4). Wolbergs found very small amounts of acid phosphatase in the prostate gland of the steer (5).

Although Gutman and Gutman (6) found very high levels of acid phosphatase in human seminal fluid, the alkaline enzyme was not present in appreciable quantities. Upon fractionating the human semen ejaculate and employing the acid enzyme of the prostatic secretion as a 'tracer', Gutman and Gutman (6) were able to demonstrate that the secretion from the prostate gland precedes that of the seminal vesicles in the order of ejaculation. Though the function of acid phosphatase is yet undetermined, the high concentration of acid phosphatase, the optimal pH of the adult human vagina and the suitable substrates existent in seminal fluid have suggested to Gutman *et al.* (3, 4, 6, 7) that the enzyme exercises some significant function in reproduction. However, the semen of sterile men contained acid phosphatase within the normal limits (6). Reid, Ward and Salsbury (8) showed that the level of alkaline phosphatase in the plasma of bulls declines linearly with an increased rate of spermatozoa production. Lardy and Phillips (9) reported the presence of alkaline phosphatase in the semen of the bull with a greater per unit volume concentration in the spermatozoa than in the seminal fluid. Very small quantities of acid phosphatase were found in the semen of the bull (10).

These reports would indicate that differences exist between species relative to the level of phosphatases in semen and that at least the acid enzyme may be involved in some manner in reproduction. It was the purpose of this investigation to study the variations in the concentration of acid and alkaline phosphatase in consecutive ejaculates of individual bulls and in the ejaculates from different bulls and to attempt to correlate these variations with various semen characteristics and diet.

Received for publication January 29, 1948.

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³ The authors are indebted to Professor C. E. Shuart and Messrs. Martin Struble and Donald Eby for the feeding and management of the bulls and many other services.

METHODS

A series of ejaculates was obtained individually with artificial vaginas from each of 10 bulls at 33 months of age and analyzed for acid (pH 5.0) and alkaline (pH 9.3) phosphatase activity according to the method of King and Armstrong (11) as modified by Wiese *et al.* (12) for blood plasma. The pH of a number of ejaculates was determined before and after incubation for one hour at 37°C. with a glass electrode Beckman pH meter, spermatozoa concentrations were determined by direct counting with a cytometer, and the initial motility of spermatozoa was estimated at 100° F. in a constant temperature stage incubator. Ejaculates were taken as long as libido persisted and each series was obtained within a 30-minute period. Although

TABLE 1. COMPOSITION OF CONCENTRATE MIXTURES

INGREDIENTS	GROUP I %	GROUP II %	INGREDIENTS	GROUP I %	GROUP II %
Ground yellow corn.....	54.0	10.0	Dehydrated alfalfa.....		10.0
Beet pulp.....	25.0		Limestone.....		2.0
Corn gluten meal.....	10.0		Iodized salt.....	1.0	1.0
Cane molasses.....	10.0	10.0	Bone meal.....		.7
Linseed meal.....		12.0	Brewers yeast.....		1.95
Soybean meal.....		17.0	Mineral salt mixture ¹1
Crushed oats.....		25.0	Fish liver oil ²2
Wheat bran.....		10.0	Irradiated yeast ³05

¹ Mineral salt mixture consisted of: $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 50%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 44.5%; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.0%; and $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.5%.

² Fish liver oil containing 15,000 I.U. or more of vitamin A per gram.

³ Irradiated yeast containing 9000 U.S.P. units of vitamin D per gram.

TABLE 2. ANALYSIS OF FEEDS ON DRY MATTER BASIS AND SUPPLEMENTS ADDED TO COMPLEX CONCENTRATE MIXTURE

FEED MIXTURE	TOTAL PROTEIN	ETHER EX- TRACT	CRUDE FIBER	ASH	Ca	P	Mn	SUPPLEMENT ADDED PER LB. FEED				
								Fe	Cu	Co	Vit. A	Vit. D
	%	%	%	%	%	%	%	mgm.	mgm.	mgm.	I. U.	U.S.P. units
Group I.....	12.33	2.34	8.42	4.35	.38	.22	.0045	0	0	0.00	0	0
Group II.....	20.51	4.80	10.84	8.28	1.18	.64	.0207	40	6	0.45	13,620	3,043
Hay.....	7.05	2.07	39.58	4.87	.29	.21	.0028	0	0	0.00	0	0

semen had been taken from these bulls at heavy rates previously, no attempt had been made to obtain semen during a period of 3.5 months prior to this experiment.

The 10 bulls used in this study constituted two feed groups described earlier (8). *Group I* received a simple unsupplemented concentrate mixture, and *group II* a complex concentrate mixture supplemented with minerals and vitamins (tables 1 and 2). Both groups received the same average grade hay (table 2).

RESULTS

Levels of phosphatases in bull semen. Some representative data obtained in this experiment are presented in table 3. Although considerable variation was found in both the alkaline and acid phosphatase levels of the ejaculates of an individual bull

and between the ejaculates of different bulls, it is significant that the activity of phosphatase in all but two cases was greater at pH 9.3 than at pH 5.0. The average levels in 56 ejaculates obtained from both groups of bulls were 393 and 170 units of alkaline and acid phosphatase, respectively, per 100 ml. semen. The relationship between the levels of acid and alkaline phosphatase was not significant. No definite pattern of phosphatase concentration accompanied the sequence of ejaculates; however, each of the first few ejaculates generally contained much more acid phosphatase than the last ejaculate taken. This phenomenon was concomitant with a decrease in spermatozoa concentration at or near the end of a series of ejaculates. In most cases, the level of alkaline phosphatase was maintained more uniformly throughout

TABLE 3. REPRESENTATIVE DATA ON THREE SERIES OF EJACULATES OBTAINED CONSECUTIVELY

BULL & GROUP NO.	EJACULATE NO.	PHOSPHATASES		SPERM. CONC.	INITIAL MOTILITY	SEMEN VOLUME	pH	
		Alkaline	Acid				Initial	Decrease upon incubation
		unit/100 ml.		10 ⁶ /mm. ³	%	ml.		
6—I	1	438.6	221.7	1.544	97	1.2		
	2	253.4	224.7	1.728	100	5.8	6.42	.41
	3	266.8	119.0	.689	60	6.0	6.69	.19
	4	228.8	47.4	.178	50	1.9		
	5	269.6	103.6	.656	57	6.5	6.59	.13
	6	134.9	69.6	.056	0	.9		
9—I	1	300.9	203.7	1.565	73	5.5	6.35	.42
	2	375.3	166.1	.965	55	6.0	6.70	.58
	3	373.1	161.2	.905	65	5.9	6.40	.23
	4	342.7	158.0	1.105	74	3.8		
	5	301.8	113.8	.765	70	7.6	6.66	.23
	6	343.9	94.4	.380	18	5.5		
13—II	1	743.9	255.9	1.411	78	5.0		
	2	172.0	338.3	2.217	97	5.7	6.41	.61
	3	3458.7	189.3	.678	55	4.5		
	4	541.9	205.0	.756	73	4.8		
	5	729.1	135.7	.048	20	3.3		

a series of ejaculates than was that of acid phosphatase (table 3, bull 9). In a few cases, such as that demonstrated in table 3 by the data for bull 13, greatly different levels of alkaline phosphatase were found within an ejaculate series.

Relationship of level of phosphatases to semen characteristics. No significant relationship of the content of semen alkaline phosphatase to the concentration of spermatozoa, initial motility, initial pH, change in pH upon incubation or volume of semen was found (table 4). The acid enzyme, however, showed some degree of relationship to these characteristics, as is indicated by the coefficients of correlation in table 4. Of these the highest degree of relationship was found between the semen acid phosphatase level and the concentration of spermatozoa. Figure 1 demonstrates the apparent dependence of the semen acid phosphatase level upon the concentration of spermatozoa.

Relationship of diet to semen phosphatases. A striking dietary influence upon the semen level of alkaline and acid phosphatase was observed (table 5). Although the level of acid phosphatase in both groups was dependent upon the concentration of spermatozoa, the enzyme was significantly higher per unit concentration of spermatozoa in the semen of bulls consuming the complex concentrate mixture than in that of bulls receiving the simple ration (table 6 and fig. 1). Tables 4 and 6 show that the coefficient of correlation is higher between the acid phosphatase level and the spermatozoa concentration of the ejaculates of the individual groups than for all animals. Calculation of the regression of spermatozoa concentration on the acid

TABLE 4. RELATIONSHIP BETWEEN SEMEN PHOSPHATASES AND CHARACTERISTICS OF THE SEMEN OF BULLS ON BOTH RATIONS

SEMEN CHARACTERISTICS	NO. CASES	SEMEN PHOSPHATASES		
		Acid		Alkaline
		Correlation coeff.	Std. error	Correlation coeff.
Spermatozoa conc.....	56	.771 ¹	±.055	.047
Initial motility.....	56	.514 ¹	±.100	.062
Semen volume.....	56	.333 ²	±.121	.008
Initial pH.....	24	-.336	±.189	.340
pH change on incubation.....	24	.458 ²	±.168	.077

¹ Significant at 1% level of probability.

² Significant at 5% level of probability.

TABLE 5. MEAN GROUP SEMEN CHARACTERISTICS

GROUP	NO. OF BULLS	NO. OF EJACULATES	PHOSPHATASE		SPERM CONC.	INITIAL MOTILITY	SEMEN VOLUME	pH		
			Alkaline	Acid				No. detr.	Initial	Decrease on incubation
			units/100 ml.		10 ⁶ /mm. ³	%	ml.			
I	5	28	307.3	141.9	.739	48	4.8	13	6.66	.25
II	5	28	477.6	198.2	.738	56	5.2	11	6.68	.32
Significance of difference between group means.....			n.s.	1	n.s.	n.s.	2		n.s.	n.s.

¹ Significant at 1% level of probability.

² Significant at 5% level.

n.s., not significant.

phosphatase level for each group indicated a straight line relationship and gave almost parallel regression lines for the two groups (fig. 1).

It will be noted in table 6 that significant coefficients of correlation existed between the acid phosphatase level and the initial motility, volume, initial pH and the fall in pH subsequent to incubation of semen from group II bulls. The same correlations for the semen of group I bulls were not mathematically significant.

DISCUSSION

The results of this study in which it was shown that the semen of the male bovine contains appreciably greater quantities of alkaline phosphatase than of the acid

enzyme are in contrast to the finding of Gutman and Gutman (6) which points out that the seminal fluid of man contains very large amounts of acid phosphatase and is

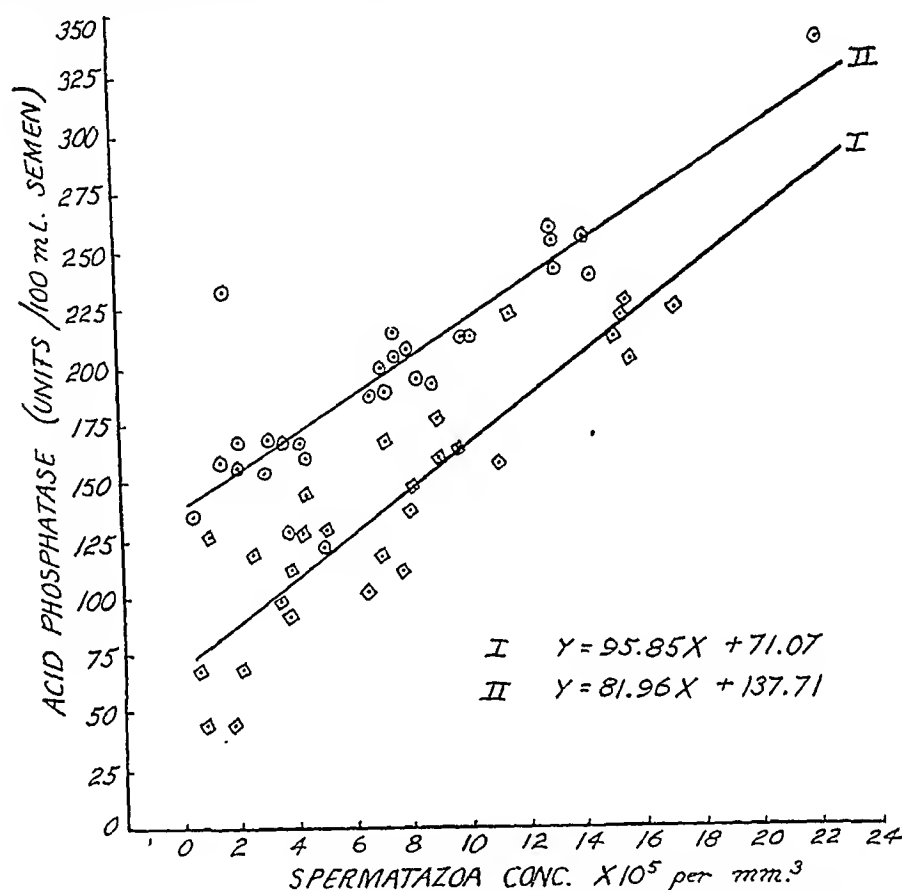


Fig. 1. REGRESSION LINES OF SPERMATOZOA CONCENTRATION on the semen acid phosphatase level of both groups of bulls. Line I, calculated for group I by the equation: $Y = 95.85X + 71.07$. Line II, calculated for group II by the equation: $Y = 81.96X + 137.71$. A line equidistant from lines I and II would represent the relationship for all ejaculates.

TABLE 6. RELATIONSHIPS OF SEMEN CHARACTERISTICS TO SEMEN ACID PHOSPHATASE LEVELS OF EACH GROUP

SEMEN CHARACTERISTICS	GROUP I			GROUP II		
	No. of ejaculates	Correlation coeff.	Std. error	No. of ejaculates	Correlation coeff.	Std. error
Spermatozoa conc.....	28	.902 ¹	.035	28	.876 ¹	.046
Initial motility.....	28	.372	.169	28	.675 ¹	.107
Semen volume.....	28	.241	.185	28	.458 ²	.155
Initial pH.....	13	-.276	.279	11	-.606 ²	.211
Decrease in pH upon incubation..	13	.101	.298	11	.688 ²	.176

¹ Significant at 1% level of probability.

² Significant at 5% level of probability.

practically devoid of alkaline phosphatase. Since the function of acid phosphatase in semen has not yet been determined, it is not known whether or not this difference is associated with differences in the metabolism of spermatozoa and general repro-

dictive physiology of these two species. The metabolic requirements of the spermatozoa of various species are known to differ, as is indicated by the effects of glycolysis inhibitors (9, 13-16) and oxidative processes (9, 17) on the motility of spermatozoa and by the variety of substrates capable of furnishing energy to spermatozoa (14, 15, 17-19). Some physiologic significance of the high level of alkaline phosphatase in bull semen seems probable on a basis similar to that suggested by Gutman *et al.* (3, 4, 6, 7) for the possible function of the high acid phosphatase of human semen.

Assuming that acid phosphatase has its origin in the prostate gland (20), the relatively small amount of acid phosphatase in the semen of the bull would indicate that the prostatic secretion comprises a small proportion of the ejaculate or that the secretion contains a low level of acid phosphatase. In view of the work of Wolberg and Pany (5), which showed that the bovine prostate gland contains only small quantities of this enzyme, the latter appears more likely. According to the contention of Gutman and Gutman (6) that the acid phosphatase originates in the prostate gland and is not secreted in appreciable amounts by other glands of the male genital tract (20), our data would seem to suggest that this gland participates to about the same extent in each of the first several ejaculates of a series obtained consecutively. On this basis, the marked drop in the acid phosphatase level of the semen obtained at or near the end of a series of ejaculates would seem to indicate also that the prostatic secretion is being progressively depleted. From our data this does not necessarily appear to be true. Because of the high correlation between the acid phosphatase level and the concentration of spermatozoa in bull semen, it appears more likely that the decline in acid phosphatase near the end of a series of ejaculates is associated with the correspondingly low spermatozoa concentration. It is not clear whether or not the spermatozoa secrete the enzyme, merely adsorb and transport it, or whether by nature the prostate gland contributes less when semen contains low spermatozoa numbers.

It was previously reported that a high correlation is found between the decline in the alkaline phosphatase of plasma and the increase in the number of spermatozoa ejaculated by bulls (8). The results of the present study showing a high level of alkaline phosphatase in semen would appear to amplify the supposition that the blood is the immediate origin of semen alkaline phosphatase and that the level of the enzyme is maintained in semen at the expense of the blood level.

Although the levels of acid and alkaline phosphatase were markedly higher in the semen of bulls receiving the complex feed than in that of bulls consuming the simple ration, the dietary factors involved were not ascertainable because of the design of this experiment. The complex concentrate mixture, however, provided 3.62 grams more Ca, 1.91 grams more P, 40 mgm. more Fe, 74 mgm. more Mn, 6 mgm. more Cu, 0.45 mgm. more Co, 37.10 grams more protein, 11.16 grams more fat, 13,620 I.U. more vitamin A and 2,043 U.S.P. units more of vitamin D per pound than was supplied in the same amount of the simple concentrate feed. The rôle of nutrition in the fertility of bulls has not been studied to any great extent. On the basis of the data presented here, the possible value of complex concentrate feeds in improving semen quality may, at least, partly depend upon the significance designated to the semen phosphatases of this species.

When all of the ejaculates from both groups was considered, the acid phos-

phatase level of semen showed varying degrees of relationship to spermatozoa functions and was directly correspondent with the concentration of spermatozoa, whereas no appreciable correlation existed between the characteristics of semen studied and the alkaline phosphatase content. When the relationships between the acid phosphatase level and the characteristics of semen were analyzed for each group separately, significant correlations were found between the acid enzyme level and the initial motility, volume, initial pH and the decline in pH upon incubation of semen from *Group II*. No significant correlation existed between the acid phosphatase concentration and these characteristics of the semen of *group I* bulls. These data, however, do not designate the significance of acid phosphatase in bull semen, although they do suggest that the level of acid phosphatase may be a valuable criterion of semen quality.

SUMMARY

1. The mean level of alkaline phosphatase was considerably higher than that of the acid phosphatase, with an average concentration of 393 (range 97 to 3459) units as compared to 170 (range 46 to 338) units of the latter per 100 ml. of bull semen.

2. The levels of both acid and alkaline phosphatase in the semen of bulls fed a complex concentrate mixture were markedly elevated above that of the semen from bulls receiving a simple concentrate mixture.

3. A high degree of relationship existed between the semen acid phosphatase level and the concentration of spermatozoa in the semen of both groups. Although the characteristics of semen from bulls receiving the complex feed showed a high correlation with the acid phosphatase level, no appreciable association of these characteristics with this enzyme's level was found in the semen produced by bulls receiving the simple ration. Also, there was no apparent relationship of the level of alkaline phosphatase in semen to any of the characteristics of semen studied.

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DOSES OF HISTAMINE PRODUCING MINIMAL AND MAXIMAL GASTRIC SECRETORY RESPONSES IN DOG AND MAN

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THE presence in the animal body of an agent which produces pharmacological actions as marked as are those of histamine raises the question whether this substance may have some function in normal physiological processes. This study of the gastric secretory response to histamine has been undertaken with the hope that the results obtained will be of value toward a solution of that question.

THE PROBLEM

Little work has been done to determine the relation of the amount of gastric gland secretion to the size of the dose of histamine injected. Rothlin and Gundlach (3) found some correlation between the amount of histamine injected subcutaneously and the volume of gastric juice response; they obtained no response to single intravenous injections. Ivy and Javois (4) noted that intravenous injection of one microgram of histamine base per kilo body weight per minute stimulated gastric secretion in a Pavlov pouch dog without causing peripheral vasodilation. Emmelin (5) reports a minimal secretory response in two Pavlov pouch dogs given $0.5 \mu\text{g}/\text{kgm}/\text{min}$. intravenously.

Obrink (6) observed the rate of secretion produced in a Heidenhain pouch dog by different rates of intravenous administration of histamine injected continuously. He observed a secretory response to $0.05 \mu\text{g}$. histamine base per kgm. per minute, and found that the dose producing a maximal response was about $1.7 \mu\text{g}$. base/kgm/min. He constructed a dose-response curve and extended it thru the origin, concluding that there was no threshold dose of histamine.

McElin and Horton (7) incidentally noted in humans that $0.03 \mu\text{g}/\text{kgm}/\text{min}$. intravenously would stimulate secretion but they felt that such a dose might not be a minimal one.

In the experiments reported here we have studied the gastric secretory response in dogs during repeated subcutaneous and continuous intravenous injection of histamine and in man during continuous intravenous injection. Our purpose was to determine the minimal and maximal effective doses of histamine and to observe the characteristics of the response of the gastric glands to this drug.

PROCEDURE

Dog. One series of experiments was performed on intact dogs some of which were trained; other experiments were on trained dogs which had been operated upon several months previously to produce vagally denervated total stomach pouches.

In the intact dogs gastric juice was collected by continuous aspiration through a Rehfuess tube inserted into the stomach. Gastric juice was collected from the dogs with a gastric pouch by the usual method. All dogs were fasted for at least 12 hours before the experiment, and, after insertion of the Rehfuess tube, the stomachs of intact dogs were lavaged with lukewarm water to remove any residuum.

Received for publication March 8, 1948.

Gastric juice was collected for a control period of one or more hours to determine the basal secretory rate. Samples were separated at intervals of five minutes or more depending upon the volume of secretion. During the control period this volume was usually so small that quantities of juice sufficient for titration were obtained only with longer sample periods. The volume of juice collected was recorded, and its free and total acidity was determined by titration of 2 cc. samples with N/40 NaOH, using Topfer's reagent and phenolphthalein as indicators.

Histamine dihydrochloride¹ was administered to dogs either subcutaneously at 10-minute intervals or by intravenous drip continuously. Measured quantities of the solid dihydrochloride crystals, dehydrated in a desiccator, were dissolved in a 0.9 per cent NaCl solution just before each experiment. Concentrations of solutions for subcutaneous injection ranged from 0.02 to 0.18 mgm. base per cc. for different animals tested and the volumes injected varied from 0.05 cc. to 6.5 cc. per 10 minutes. With the larger volumes, the dose was divided and injected into separate sites to insure rapid absorption. Intravenous injections were made through a needle inserted into the foreleg vein of the dog, or, in some instances, via plastic tubing inserted into the jugular vein. The concentrations of solutions for intravenous injection ranged from 0.0002 to 0.002 mgm. base per cc., and rates of administration varied from 5 to over 600 cc. per hour. The rate of injection was controlled by observation of the drop rate in a calibrated drop-counting chamber.

Man. Experiments on humans were conducted in much the same manner as those on dogs. Subjects fasted for 12 hours before the experiment. A Reh fuss tube was inserted and after gastric lavage with water continuous aspiration of gastric juice was made, using a large syringe. Following a control period, histamine dihydrochloride was administered by continuous intravenous drip through a needle into a forearm vein. Solution concentrations for different subjects ranged from 0.0001 to 0.002 mgm. base per cc., and rates of injection varied from 10 to 550 cc. per hour.

In some experiments on humans, measurements of forehead temperatures were taken at intervals with a skin thermometer. Dogs and particularly humans were observed for side effects such as flushing, headache or nausea.

The general procedure for determination of the response to various doses of histamine was as follows. Administration of histamine was started, giving amounts known from earlier experiments to be in the range of the lowest effective doses. The dose was then increased by a factor of two when no response occurred to a subminimal dose within one hour or when no further increase in response occurred with the lower dose.

When the secretory volume was low, a response to histamine produced a rise in the acid concentration of gastric juice sometimes without significantly changing the volume rate of secretion. Also the volumes of collection samples varied considerably at times, perhaps due to irregular contractions of the gastric pouches or to

¹ Imido-Roche. The histamine used in these studies was generously supplied by the Hoffman-La Roche Co.

occasional incomplete aspiration of juice in intact dogs and man. For these reasons, in most instances we used the increase in acid concentration as an index of a response to histamine.

Determination of the minimal and maximal responses was somewhat arbitrary. A rise in gastric juice acidity of 10 or more mEq. HCl per liter over basal levels was termed a minimal response to histamine, and the dose producing that response, a minimal or threshold dose. A maximal response was assumed when no or only an insignificant increase (less than 2 or 3 mEq. HCl per liter) in acid concentration was observed to a dose of histamine twice as great as one which had produced a response.

RESULTS

Dog. Control levels of gastric secretion were very low in the pouch dogs. Often there was no free acid in the gastric juice and the total acid concentration was less than 25 mEq. HCl per liter in all but one dog, the average for all 7 dogs being 13 mEq. per liter. The rate of secretion was usually less than 5 cc. per hour (table 2). In response to large doses of histamine, the total acidity rose to as high as 155 mEq. of HCl per liter, averaging about 140, and the volume rate increased to values averaging over 70 cc. per hour (table 3).

Generally the basal acidity and rate of secretion was higher in the intact dogs than in the pouch dogs (table 4), but the responses to large doses of histamine were similar in the two types of dogs, whether the histamine was administered subcutaneously or intravenously (table 5).

This indicates that the larger volumes of solution injected by the intravenous method did not affect the rate of gastric secretion, nor would such be expected, since the higher volume rates were not injected for more than 20 or 30 minutes.

A. POUCH DOGS GIVEN HISTAMINE SUBCUTANEOUSLY AT 10-MINUTE INTERVALS. The method of determination of the minimal and maximal effective doses of histamine can be shown by the description of an experiment on the vagally denervated total pouch dog no. 2, in which histamine was injected subcutaneously at 10-minute intervals (fig. 1).

After a control period of 1.5 hours, drug administration was begun. Every 10 minutes 0.25 μ g. of histamine base² was injected, an equivalent of 0.025 μ g/kgm/min., assuming that all the 0.25 μ g. was absorbed within each 10-minute period.

Within 35 minutes after starting the drug, total acidity rose from 3 to 16 mEq. HCl per liter. The volume increase was negligible. Although it would have been more desirable to start with a subminimal dose, the response in this case was small enough so that we may say with some certainty that the histamine dose producing a minimal response in this dog was in the range of 0.025 μ g/kgm/min.

Very soon the response began to decrease and after 70 minutes the dose was doubled to equal 0.05 μ g/kgm/min., after which a further increase in acidity and secretory rate occurred. In a similar manner the dog was given successively larger doses of histamine at intervals determined by the response. The acid response to a dose of 3.2 μ g/kgm/min. was no greater than that observed with a dose of 1.6 μ g.

² All doses are given in terms of histamine base.

and so it was established that the dose of histamine producing a maximal response in pouch dog no. 2 for this experiment was about $1.6 \mu\text{g./kgm./min.}$

The minimal effective dose of histamine injected subcutaneously every 10 minutes was determined for 7 pouch dogs. Reference to tables 1 and 2 will show that this minimal dose ranged from 0.02 to $0.09 \mu\text{g./kgm./min.}$, the average being 0.03 .

The maximal effective dose of histamine injected subcutaneously every 10 minutes, as determined by the acid concentration, was found to range in seven dogs from 0.32 to $1.6 \mu\text{g./kgm./min.}$; the average was $0.75 \mu\text{g./kgm./min.}$ (tables 1 and 3A).

Analysis of the data revealed that in some cases the volume rate of secretion was still increasing to doses which produced no further significant increase in acid concentration. This was to be expected, since Gray (8) has shown that the relation of acid concentration to the rate of secretion is hyperbolic, and arbitrarily we have

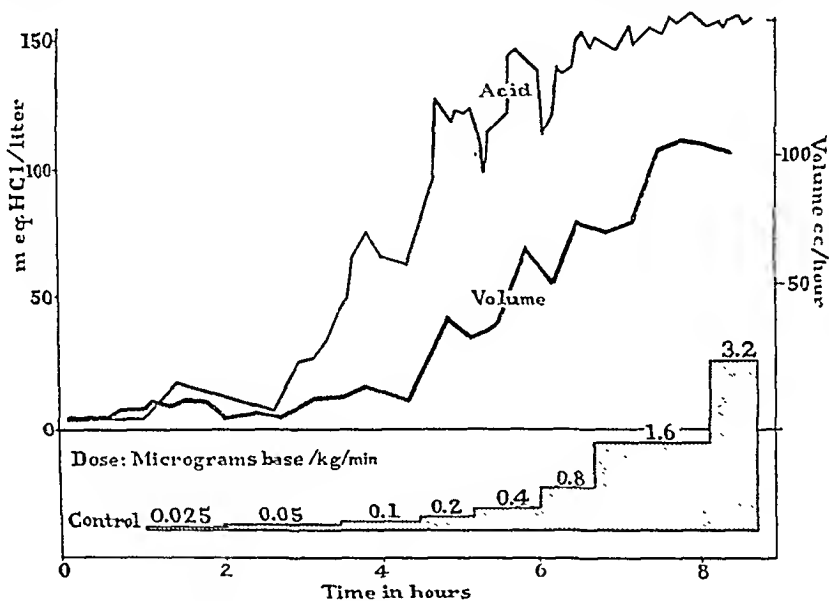


Fig. 1. GASTRIC RESPONSE of total pouch dog (#8) to subcutaneous histamine

considered a response to be maximal when doubling a dose of histamine did not increase the acid concentration by more than 2 or 3 mEq. HCl per liter. The acid output is a better criterion of the parietal cell response to histamine, and therefore the dose producing the maximal acid output in mEq. HCl per hour was also determined.

The maximal subcutaneous dose for pouch dogs in terms of acid output was obtained in 7 dogs. It ranged from 0.38 to $1.6 \mu\text{g./kgm./min.}$, with an average of 1.0 (tables 1 and 3B). This average dose is somewhat greater than that determined by measure of the acid concentration, although in some dogs there was no difference in the dose using the two criteria of maximal response.

B. POUCH DOGS GIVEN CONTINUOUS INTRAVENOUS HISTAMINE. The minimal effective intravenous dose was determined in 5 pouch dogs. It ranged from 0.02 to 0.08 micrograms per kilogram per minute, the average value being 0.045 (tables 1 and 2). These values compare with the average threshold dose of $0.03 \mu\text{g./kgm./min.}$ by the subcutaneous method of histamine injection into pouch dogs, and it is

unlikely that the difference between the subcutaneous and intravenous minimal effective dose is significant.

The maximal dose of continuous intravenous histamine with respect to the acid concentration of gastric juice was found in 6 pouch dogs to range from 0.16 to 2.6

TABLE I

A. HISTAMINE DOSE FOR MINIMAL GASTRIC RESPONSE IN VAGALLY-DENERVATED TOTAL-POUCH DOGS AND IN INTACT DOGS
(micrograms histamine base per kilogram per minute)

POUCH DOGS			INTACT DOGS		
Dog No.	S.C. ¹	I.V. ²	Dog no.	S.C.	I.V.
1	0.037		IX-12	0.04	
2	0.025	0.04	IX-15	0.02	
4	0.02	0.02	IX-16	0.02	
5	0.02		IX-17	0.04	
7	0.09		IX-14		0.02
9	0.02	0.044	IX-31		0.07
11	0.02		IX-31		0.02
12		0.08	IX-33		0.08
13		0.04	IX-33		0.04
Average.....	0.03	0.045		0.03	0.045

B. HISTAMINE DOSE FOR MAXIMAL GASTRIC RESPONSE (ACID CONCENTRATION AND RATE OF ACID OUTPUT) IN VAGALLY DENERVATED TOTAL POUCH DOGS AND IN INTACT DOGS

POUCH DOGS						INTACT DOGS					
Subcutaneous injection			Intravenous injection			Subcutaneous injection			Intravenous injection		
Dog no.	Acid conc.	Acid output	Dog no.	Acid conc.	Acid output	Dog no.	Acid conc.	Acid output	Dog no.	Acid conc.	Acid output
1	1.2	1.2	1	0.4	1.6	IX-12	0.62	1.2	IX-2	1.4	2.8
2	1.6	1.6	2	2.6	2.6	IX-15	0.32	0.64	IX-6	2.7	2.7
4	0.38	0.38	4	0.16	0.64	IX-16	0.64	0.64	IX-19	1.6	3.2
4	0.32	0.64	8	0.64	1.3	IX-17	1.3	1.3	IX-21	1.6	1.6
5	0.67	1.3	9	0.17	0.32						
7	0.73	1.5	11	0.64	1.3						
9	0.32	0.64									
Average...	0.75	1.0		0.77	1.3		0.72	0.94		1.8	2.6

¹ Subcutaneous.

² Intravenous.

$\mu\text{g}/\text{kgm}/\text{min.}$, the average being $0.77 \mu\text{g}$. The range of maximal doses was somewhat greater by intravenous injection than by subcutaneous injection, but the average dose by the two methods was the same (tables 1 and 3A). The maximal dose in terms of hourly output of HCl ranged in the 6 pouch dogs from 0.32 to 2.6 $\mu\text{g}/\text{kgm}/\text{min.}$ The average dose was $1.3 \mu\text{g}/\text{kgm}/\text{min.}$, nearly twice the average maximal dose determined by a measure of the acid concentration (tables 1 and 3B).

C. INTACT DOGS GIVEN SUBCUTANEOUS HISTAMINE AT 10-MINUTE INTERVALS. The minimal effective subcutaneous dose for intact dogs ranged from 0.02 to 0.04 $\mu\text{g}/\text{kgm}/\text{min.}$ in 4 dogs, 0.03 being the average (tables 1 and 4).

The maximal effective dose in intact dogs by subcutaneous injection, using acid concentration as an indication of response, ranged from 0.32 to 1.3 $\mu\text{g}/\text{kgm}/\text{min.}$ in 4 dogs, with an average of 0.72 $\mu\text{g.}$ (tables 1 and 5A). Doses producing maximal acid output in these 4 dogs ranged from 0.64 to 1.3 $\mu\text{g}/\text{kgm}/\text{min.}$, averaging 0.94 $\mu\text{g.}$, a value not appreciably larger than that established using acid concentration as a criterion of response (tables 1 and 5B).

TABLE 2. HISTAMINE DOSE FOR MINIMAL GASTRIC RESPONSE IN VAGALLY DENERVATED TOTAL POUCH DOGS

EXP. NO.	DOG. NO.	CONTROL			SUBMINIMAL				MINIMAL				VOL.
		mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.			
		Free	Total			Free	Total			Free	Total		
Subcutaneous Injection													
				cc./hr.				cc./hr.				cc./hr.	
1	1	15	22	2					0.037	44	65	7	
2	2	0	3	6					0.025	8	16	8	
3	4	5	22	3	0.01	6	27	4	0.02	35	57	7	
4	5	0	3	2					0.02	3	15	5	
5	7	0	14	5	0.046	5	16	6	0.09	27	33	8	
6	9	0	5	2					0.02	18	35	8	
7	11	8	17	4	0.01	8	18	5	0.02	22	34	6	
Average....		4	12	3	0.022	6	20	5	0.033	22	36	7	
Intravenous Injection													
1	2	4	20	3	0.02	5	14	3	0.04	29	46	7	
2	4	0	8	7					0.02	24	41	5	
3	9	0	10	1	0.026	0	16	4	0.044	65	82	11	
4	12	0	3	2	0.04	0	5	1.4	0.08	48	79	16	
5	13	4	26	1.6	0.02	2	18	2	0.04	11	27	4	
Average....		2	13	3	0.026	2	13	3	0.045	35	55	9	

¹ Micrograms of histamine base per kilogram per minute.

D. INTACT DOGS GIVEN CONTINUOUS INTRAVENOUS HISTAMINE. The minimal effective intravenous dose ranged in the 6 intact dogs studied from 0.02 to 0.08 $\mu\text{g}/\text{kgm}/\text{min.}$, averaging 0.045 (tables 1 and 4). This compares well with threshold dose values obtained by subcutaneous injection of intact dogs and either subcutaneous or intravenous injection of pouch dogs. From the data at hand it seems unlikely that there is any significant difference between the threshold dose for vagotomized total pouch dogs and that for intact dogs, nor does there appear to be any difference in the minimal dose whether histamine is administered subcutaneously at ten-minute intervals, or continuously by the intravenous method.

The maximal effective dose of intravenous histamine was found in four intact dogs to range from 1.4 to 2.7 $\mu\text{g/kgm/min.}$ with an average of 1.8 (tables 1 and 5A). This value was obtained using the acid concentration as a criterion of response. The maximal intravenous dose in terms of total hourly acid output ranged from 1.6 to 3.2 $\mu\text{g.}$ in the four dogs, averaging 2.6 $\mu\text{g/kgm/min.}$ (tables 1 and 5B).

The average maximal dose in intact dogs was about twice as large by intravenous injection as was the average maximal dose in pouch dogs by the same method. If this difference is significant it is difficult to explain. It may be that vagal impulses

TABLE 3A. HISTAMINE DOSE FOR MAXIMAL GASTRIC RESPONSE (ACID CONCENTRATION) IN VAGALLY DENERVATED TOTAL POUCH DOGS

EXP. NO.	DOG NO.	BELOW MAXIMAL				MAXIMAL				ABOVE MAXIMAL			
		Dose ¹	mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.
			Free	Total			Free	Total			Free	Total	
Subcutaneous Injection													
					cc./hr.				cc./hr.				cc./hr.
1	1	0.59	134	140	59	1.2	139	144	64	2.4	139	144	62
2	2	0.80	142	146	75	1.6	151	155	103	3.2	150	154	104
3	4	0.19	144	149	47	0.38	149	152	55	0.76	141	146	57
4	4	0.16	121	132	50	0.32	133	144	70	0.64	131	139	80
5	5	0.33	119	124	37	0.67	139	141	78	1.3	138	141	102
6	7	0.36	131	135	49	0.73	134	140	56	1.5	135	140	83
7	9	0.16	130	137	49	0.32	133	139	57	0.64	137	141	77
Average...		0.38	122	138	52	0.75	140	145	69	1.5	138	144	81
Intravenous Injection													
1	1	0.2	128	136	30	0.4	136	144	45	0.8	136	144	60
2	2	1.3	125	131	116	2.6	128	135	122	4.0	126	132	87
3	4	0.08	122	133	26	0.16	128	136	21	0.32	117	125	69
4	8	0.32	94	114	33	0.64	105	121	42	1.3	114	124	51
5	9	0.08	109	121	21	0.17	124	134	31	0.32	124	131	40
6	11	0.32	114	120	76	0.64	135	139	118	1.3	135	139	162
Average...		0.38	116	126	50	0.77	126	132	61	1.3	125	133	78

¹ Micrograms of histamine base per kilogram per minute.

caused by nausea to histamine, or enterogastric reflexes caused by acid entering the intestine, somewhat delayed the maximal response of the gastric glands to histamine in the intact dogs. However, subcutaneous maximal doses for intact dogs were no larger than those for pouch dogs. On the basis of the results of the present experiments, we are inclined to believe that this apparent difference is not a significant one. In future experiments to test this, the variation of the maximal doses of histamine in different dogs should be considered.

Retching was observed in three pouch dogs with doses of 0.08, 3.2 and 4 $\mu\text{g/kgm/min.}$ One intact dog became dyspneic to a dose of 0.32 $\mu\text{g.}$, and another intact dog showed marked flushing to the very large dose of 12.5 $\mu\text{g/kgm/min.}$ This dog was dazed at the completion of the experiment as were several others given large

doses of histamine. Another observation was an uneasiness in several dogs when the dose was increased. This sign may have been an expression of nausea or headache in the animals.

Man. Measurement of the gastric acidity and secretory rates in humans during the control period revealed two definite groups. The subjects in one group secreted volumes of juice of around 30 cc. per hour containing about 20 mEq. HCl per liter, while in the other group rates of about 90 cc. per hour and acid concentrations averaging 85 mEq. HCl per liter were observed to occur.

TABLE 3B. HISTAMINE DOSE FOR MAXIMAL GASTRIC RESPONSE (RATE OF ACID OUTPUT) IN VAGALLY DENERVATED TOTAL POUCH DOGS

EXP. NO.	DOG NO.	BELOW MAXIMAL			MAXIMAL			ABOVE MAXIMAL		
		Dose ¹	Vol.	mEq. HCl/hr.	Dose ¹	Vol.	mEq. HCl/hr.	Dose ¹	Vol.	mEq. HCl/hr.
<i>Subcutaneous Injection</i>										
			cc. hr.			cc. hr.			cc. hr.	
1	1	0.59	59	8.3	1.2	64	9.2	2.4	62	8.9
2	2	0.8	75	11.	1.6	103	16.	3.2	104	16.0
3	4	0.19	47	7.	0.38	55	8.4	0.76	57	8.3
4	4	0.32	70	10.1	0.64	80	11.	1.3	84	11.5
5	5	0.67	78	11.	1.3	102	14.4			
6	7	0.73	56	7.8	1.5	83	11.6			
7	9	0.32	57	7.9	0.64	77	10.9	1.3	78	10.9
Average.....		0.52	63	9.	1.0	81	11.6	1.8	77	11.1
<i>Intravenous Injection</i>										
1	1	0.8	60	8.8	1.6	80	10.6			
2	2	1.3	116	14.8	2.6	122	16.4	4.0	87	11.5
3	4	0.32	69	8.6	0.64	90	11.2	1.3	92	11.7
4	8	0.64	42	5.1	1.3	51	6.3			
5	9	0.17	51	4.1	0.32	40	5.2			
6	11	0.64		16.4	1.3	162	22.5			
Average.....		0.65	53	9.6	1.3	91	12.	2.6	90	11.6

¹ Micrograms of histamine base per kilogram per minute.

Usually only small doses of histamine were given the human subjects, but when larger amounts were given, total acidity of 140 mEq. HCl per liter and secretory rates of over 250 cc. per hour were recorded.

The minimal dose of continuous intravenous histamine which produced a gastric secretory response was determined in nine experiments on 6 human subjects. The results shown in table 6 range from 0.001 to 0.006 $\mu\text{g}/\text{kgm}/\text{min.}$ except for one subject, no. 7, K. K., who had a high basal acidity and showed a response only with the much higher dose of 0.016 $\mu\text{g}/\text{kgm}/\text{min.}$ The average minimal dose was 0.004 micrograms per kilogram per minute. Figure 2 shows the very definite response to 0.002 $\mu\text{g}/\text{kgm}/\text{min.}$

There was apparently no difference in the minimal effective dose between sub-

TABLE 4. HISTAMINE DOSE FOR MINIMAL RESPONSE IN INTACT DOGS

EXP. NO.	DOG NO.	CONTROL			SUBMINIMAL				MINIMAL			
		mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.
		Free	Total			Free	Total			Free	Total	
Subcutaneous Injection												
				cc./hr.				cc./hr.				cc./hr.
1	IX-12	85	95	25					0.04	112*	128	32
2	IX-15	0	8	3					0.02	16	24	6
3	IX-16	0	8	7					0.02	20	34	35
4	IX-17	0	14	10	0.02	8	16	4	0.04		48	5
Average.....		21	31	11					0.03	49	58	20

Intravenous Injection

1	IX-14	35	65	11					0.02	90	100	21
2	IX-31	82	103	3	0.04	11	40	15	0.07	116	124	60
3	IX-31	20	40	10					0.02	94	105	12
4	IX-33	30	62	4	0.04	0	16	5	0.08	49	68	10
5	IX-33	0	5	3					0.04	34	64	22
6	IX-34	0	34	8	0.02	0	22	13	0.04	114	124	36
Average.....		28	52	6	0.03	4	26	11	0.045	83	98	27

¹ Micrograms of histamine base per kilogram per minute.

TABLE 5A. HISTAMINE DOSE FOR MAXIMAL GASTRIC RESPONSE (ACID CONCENTRATION) IN INTACT DOGS

EXP. NO.	DOG NO.	BELOW MAXIMAL				MAXIMAL				ABOVE MAXIMAL			
		Dose ¹	mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.
			Free	Total			Free	Total			Free	Total	
Subcutaneous Injection													
					cc./hr.				cc./hr.				cc./hr.
1	IX-12	0.32	124	128	33	0.62	137	141	61	1.2	138	143	74
2	IX-15	0.16	124	128	47	0.32	132	136	91	0.64	134	138	102
3	IX-16	0.32	87	90	25	0.64	105	110	39	1.3	110	114	38
4	IX-17	0.64	105	112	39	1.3	141	144	81				
Average...		0.36	110	114	36	0.72	129	133	68	1.0	127	132	71

Intravenous Injection

5	IX-2					1.4	133	139	77	2.8	137	141	88
6	IX-6	1.4	132	136	91	2.7	143	146	90	3.8	137	141	71
7	IX-19					1.6	136	144	90	3.2	131	143	102
8	IX-21	0.8	136	141	49	1.6	143	147	72	3.2	142	148	69
Average...		1.1	134	138	70	1.8	129	144	82	3.2	137	143	82

¹ Micrograms of histamine base per kilogram per minute.

TABLE 5B. HISTAMINE DOSE FOR MAXIMAL GASTRIC RESPONSE (RATE OF ACID OUTPUT IN INTACT DOGS)

EXP. NO.	DOG NO.	BELOW MAXIMAL			MAXIMAL			ABOVE MAXIMAL		
		Dose ¹	Vol.	mEq. HCl/hr.	Dose ¹	Vol.	mEq. HCl/hr.	Dose ¹	Vol.	mEq. HCl/hr.
Subcutaneous Injection										
			cc./hr.			cc./hr.			cc./hr.	
1	IX-12	0.62	61	8.6	1.2	74	10.1			
2	IX-15	0.32	91	12.	0.64	102	13.2			
3	IX-16	0.32	25	2.3	0.64	39	4.2	1.3	38	4.1
4	IX-17	0.64	39	4.4	1.3	81	11.7			
Average.....		0.48	54	6.8	0.94	74	9.8			

Intravenous Injection

1	IX-2	1.4	77	9.8	2.8	88	11.7	5.6	79	10.7
2	IX-6	1.4	91	10.8	2.7	90	12.5	3.8	71	9.6
3	IX-19	1.6	90	12.2	3.2	102	14.2			
4	IX-21	0.8	49	6.9	1.6	72	10.3	3.2	69	10.
Average.....		1.3	77	9.9	2.6	88	12.2	4.2	73	10.1

¹ Micrograms of histamine base per kilogram per minute.

TABLE 6. INTRAVENOUS HISTAMINE DOSE FOR MINIMAL GASTRIC RESPONSE, HEADACHE AND FLUSH IN HUMANS

EXP. NO.	SUBJECT	CONTROL			MINIMAL					
		mEq. HCl/l.		Vol.	Dose ¹	mEq. HCl/l.		Vol.	Headache	Flush
		Free	Total			Free	Total			
				cc./hr.				cc./hr.		
1	M. G.								0.04	0.04
2	M. G.	0	15	35	0.002	12	29	54	0.008	
3	M. G.	0	15	48	0.002	19	48	58	0.002	
4	M. H.	10	31	30	0.006	30	54	35	0.08	0.08
5	M. H.	20	25	25	0.002	92	99	45	0.002	
6	M. H.								0.002	
7	K. K.	75	85	65	0.016	85	95	80	0.04	
8	K. K.	10	30	25	0.002	20	40	30		
9	W. S.	60	75	60	0.002	105	120	60		
10	C. M.	65	75	105	0.001	90	100	130		0.001
11	A. R.	70	80	45	0.004	100	110	95		
12	V. R.									
Average.....		34	46	49	0.004	61	77	65	0.02	0.047

¹ Micrograms of histamine base per kilogram per minute.

jects with low and those with high basal acidity except in one case. *Subject K. K.* in one experiment had a low basal acidity and showed a gastric response to a dose of 0.002 $\mu\text{g}/\text{kgm}/\text{min}$. In another experiment when his basal acidity and volume

were high, he responded only to a dose of $0.016 \mu\text{g/kgm/min}$. The explanation may be that in the second experiment the subject was secreting at near maximal levels and was therefore less responsive to the lower doses of histamine.

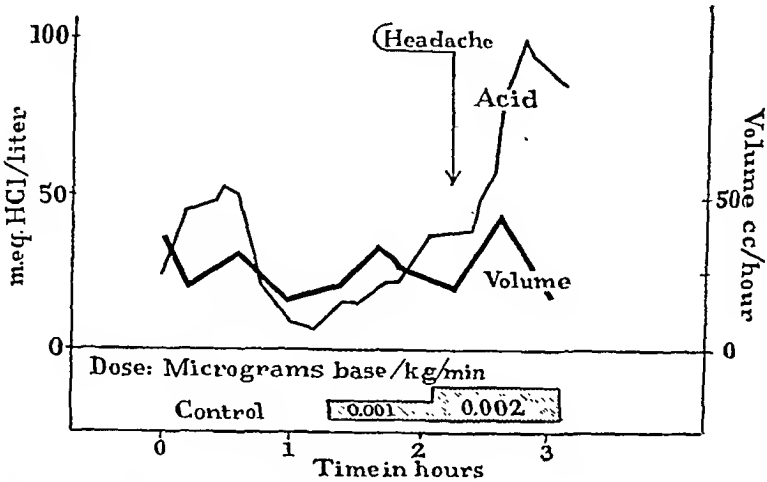


Fig. 2. GASTRIC RESPONSE of human (M. H.) to intravenous histamine

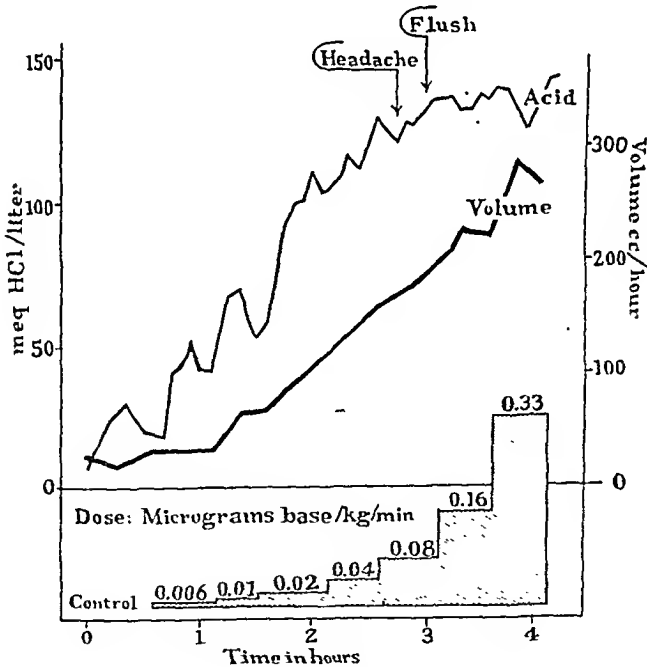


Fig. 3. GASTRIC RESPONSE of human (M. H.) to intravenous histamine

Table 6 shows the doses producing headache in nine experiments on 4 subjects. They ranged from 0.002 to $0.08 \mu\text{g/kgm/min}$. It can be seen that there was considerable variation in the headache-producing dose in different subjects and in repeated experiments on the same subject. Headaches sometimes occurred within a few minutes after a dose increase, but this was not always true. Headaches occurred in two subjects without a gastric secretory response and in two other experiments the

headache accompanied the gastric secretory response. Flushing occurred in four subjects to doses of 0.001 to 0.08 $\mu\text{g}/\text{kgm}/\text{min.}$, and forehead temperatures rose from 0.1 to 1.6° C. in 8 of 9 subjects.

No strict correlation between flushing, headache, forehead temperature and gastric secretion was apparent, except that a gastric secretory response preceded flushing or skin temperature rise and either preceded or accompanied a headache. In interpreting the relation of a gastric response and headache, the possibility should be considered that fasting and a tube in the stomach may decrease the headache threshold.

The maximal dose in humans was not determined. Gastric acidity and rate of secretion were still increasing in one subject given 0.18 $\mu\text{g}/\text{kgm}/\text{min.}$ In another subject it appeared that a maximal acid concentration was obtained with a dose of 0.16 $\mu\text{g}/\text{kgm}/\text{min.}$ (fig. 3). A secretory rate of 285 cc. per hour occurred in this subject with a dose of 0.33 $\mu\text{g}/\text{kgm}/\text{min.}$ but it is not certain that this was the maximal secretory rate.

DISCUSSION

The doses of histamine established as producing minimal and maximal gastric secretory responses in dogs and man are admittedly approximations, since the assignment of dosage for these responses was often arbitrary. As previously mentioned, an increase of 10 or more mEq. HCl per liter was assumed to be a minimal response. There was sometimes a latent period of one half hour or more after starting histamine until a response was observed, probably due either to a conditioning of the parietal cell to the increased plasma histamine concentration or to a gradual rise of the plasma histamine concentration to an effective level. It was noted that with near threshold doses of histamine the acid concentration after a temporary rise often tended to return to basal levels. The significance of this will be discussed later. Care was taken particularly with intact dogs and humans to prevent the confusion of a minimal response with spontaneous fluctuations in acidity or volume. It is possible that the average threshold dose may be slightly high, the accuracy of the measure of response being a limiting factor.

Similarly, difficulty arose in the determination of maximal response doses which were assumed when doubling the histamine dose did not increase the acidity by more than 2 or 3 mEq. HCl per liter, or the acid output by 5 per cent or more. Since supramaximal doses were not always determined with respect to rate of acid output, the average values for maximal doses may be low. However, we feel that a practical measure of the range of response of the gastric glands to histamine has been established in the dog and that the minimal dose has been shown for man.

The similarity of the average threshold doses in vagotomized pouch and intact dogs by either subcutaneous or intravenous histamine injection has been pointed out; they are between 0.03 and 0.045 $\mu\text{g}/\text{kgm}/\text{min.}$

The fact that the vagotomized pouch of the entire stomach and the intact stomach show essentially the same response to histamine indicates that vagotomy does not alter the response to histamine in the dog. It is now well established that vagotomy produces a marked depression in the response to histamine in human peptic ulcer patients (20). Unless peptic ulcer patients differ from normal subjects in

showing a decreased responsiveness to histamine after vagotomy, we must conclude that vagotomy produces different effects in man and in the dog.

The demonstration that injection of a certain amount of histamine into the subcutaneous tissue periodically is just as effective in stimulating gastric secretion as injection of the same amount of histamine intravenously indicates that no significant amount of inactivation of histamine occurs during its absorption from the subcutaneous tissue into the bloodstream.

It should be noted that the average threshold dose in the humans with intravenous histamine, only $0.004 \mu\text{g}/\text{kgm}/\text{min.}$, is about one-tenth that of the threshold dose for dogs.

The extreme sensitivity of the parietal cell to histamine is of interest. Weiss, Robb and Ellis (9) found that in 11 human subjects flush occurred to continuous intravenous histamine doses of from 0.03 to $0.1 \mu\text{g. base per kgm}/\text{min.}$, the average being about 0.05 . These values compare well with ours and show that the sensitivity of the parietal cell to histamine is much greater than that of the cutaneous facial vessels. They also found from responses to single intravenous doses of histamine that the cerebral vessels were about twice as sensitive as were the facial vessels. Our average headache-producing doses, assuming that headaches are associated with cerebral vessel changes (10), confirm these findings on the relative sensitivity of the cerebral vessels. These doses are often larger than the doses producing the gastric secretory response. It appears therefore that the parietal cell response is the most sensitive to histamine of any in the intact body, equalled only in some cases by the response in the cerebral vessels.

We have plotted for several pouch dogs the highest acid concentrations obtained in response to various doses of histamine as ordinates on a graph, with the log of the dose along the abscissa. An S-shaped curve was produced (fig. 4).

The ratio of the average dose producing 16 per cent of maximal response ($0.02 \mu\text{g}/\text{kgm}/\text{min.}$) to that producing an 84 per cent response ($0.2 \mu\text{g}/\text{kgm}/\text{min.}$) is one tenth, a considerably larger value than that for the same percentage range of effect by acetylcholine in slowing the heart of the eserized cat, the ratio in this instance being one thirty-fifth (11). It is an unsettled question whether the response to acetylcholine is of the graded or all-or-none type. The usual interpretation (11) of an S-shaped curve such as in figure 8 is that the dose-response relationship is of an all-or-none nature, which, in the case of gastric secretion, means that there is no secretion by a parietal cell until an effective threshold of histamine is reached, but then the cell secretes at its maximal rate. This curve represents then the variation in sensitivity to histamine within the parietal cell population. Variation in parietal cell sensitivity to histamine has been demonstrated in the isolated frog gastric mucosa. Acid secretory activity may occur in scattered gland pits while intervening glands are inactive, as determined by vital staining with neutral red (Grossman, M. I., unpublished data).

It is not necessary to assume that the two clear-cut modes of response (graded and all-or-none) are mutually exclusive and we suggest that an increase in the secretory rate of the individual parietal cell may occur, although variation in the parietal cell threshold to histamine is probably of greater importance in determining the characteristics of the response.

The manner in which histamine causes parietal secretion is of interest. One conception of HCl secretion is that it is an alteration by the parietal cell of plasma fluid which filters into and through the cell, and that the rate of secretion is determined by the blood pressure and degree of dilatation of the capillaries. The secretory response to histamine might then be due to its vasodilating action on the capillaries in the gastric mucosa. It is true that secretion is dependent on an adequate blood supply and that filtration plays an important part in the secretory process. Nevertheless it appears that histamine has some specific action on the parietal cell. The fact that Benadryl does not reduce the parietal cell response to histamine (12), although it reduces vasodilation, indicates such a specific action. A variation in the degree of vasodilatation over the dose range mentioned above to produce the in-

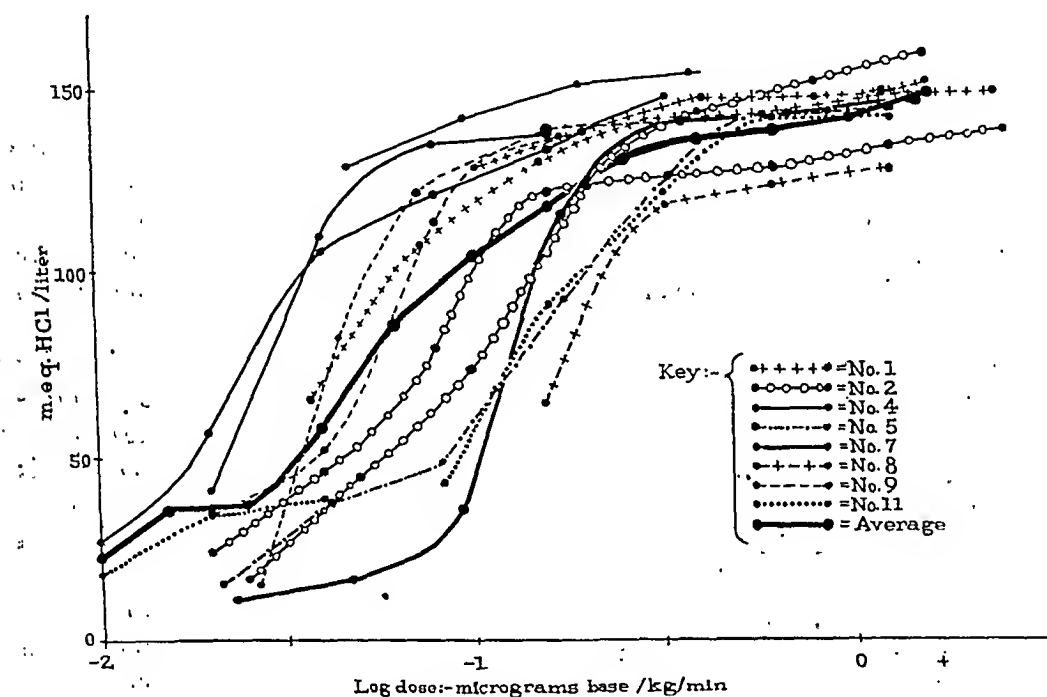


Fig. 4. GASTRIC RESPONSE of total pouch dogs to subcutaneous or intravenous histamine.

creasing rates of secretion seems unlikely. Also there are many drugs which cause vasodilatation but do not affect acid secretion. It is likely that histamine causes acid secretion mainly by a direct effect on the parietal cell.

An incidental observation in our experiments is worthy of mention. Reference to figure 1 shows that after a maximal response to a given histamine dose, the acidity started to drop until a larger dose was given to produce a further increase in response. This dog apparently showed some 'adaptation' not only to the minimal dose but to all the doses until the dose of 1.6 μ g. was reached. This phenomenon that we refer to as 'adaptation' was observed in some of the other pouch dogs and in some intact dogs and human subjects (fig. 3). The explanation of this phenomenon is not clear. In some instances in the intact dogs, the fluctuation in acidity was due to the entero-gastric regurgitation of bile-stained duodenal secretions. This could not occur in the pouch dogs. The decrease in acidity was sometimes associated with a rise in

volume, but in other cases the volume also decreased. This observation may be explained as a return of the capillary tonus to normal, thereby removing this partial effect on secretion. Emmelin (1) observed in anesthetized cats that if a histamine dose were increased slowly, the drop in blood pressure was small, and that with a constant dose the blood pressure returned to normal. We have found that this adaptation to the secretory stimulation is not complete, since gastric analysis in dogs given histamine continuously for over two weeks revealed high acid concentrations in the gastric juice.

A physiological rôle for histamine as a gastric secretory excitant

If histamine functions as a physiological excitant for gastric secretion, it might act in one of two ways. It might be released into the blood stream and serve this function as a humoral agent, or it might be released in direct contact with the parietal cell and exert its effect locally. We will consider these possibilities in order. In 1906, Edkins (13) reported that acid perfusion of the stomach remainder caused secretion of a dog's gastric pouch, and he extracted from the pyloric mucosa a substance which when injected intravenously produced a secretory response in the pouch. He concluded that this substance was a hormone for gastric secretion and he called it 'gastrin'. Edkins' evidence was inadequate for his conclusions, but subsequent experimentation has established that a hormonal mechanism does exist, although the nature of this humoral agent is not known (21).

Sacks, Ivy, Burgess and Van Dolah (14) demonstrated the presence of histamine in 'gastrin' preparations. They noted that the vasodepressor and secretory properties of the extract paralleled each other and that histaminase inactivated the preparations. They concluded that gastrin was histamine, or that there was no gastric hormone, or that it had not been isolated by the methods of preparation. Through a misinterpretation of these results, the identity of gastrin and histamine has been generally assumed in physiology textbooks.

Komarov (15), Harper (16) and Uvnas (17), by somewhat different procedures, have made extracts of hog pyloric mucosa which stimulate gastric secretion when injected intravenously into the anesthetized cat but do not have vasodepressor properties, indicating that histamine, as such, is not the active principle. We have confirmed these observations on unanesthetized pouch dogs. Preparations are not always effective, and Uvnas found that cats often become partially refractory to the extract. However, it appears certain that there is a principle which is not histamine which can be extracted from the pyloric mucosa and will stimulate gastric secretion when injected into cats or dogs. Whether this substance is the gastric hormone is not known. Further study of the problem is indicated.

Sacks *et al.* (14) were unable to detect any difference in the vasodepressor and gastric secretory activity of blood extracts or dialysates collected during fasting and digestion. MacIntosh (18) similarly could not detect any increase in blood histamine during digestion or vagal stimulation. No determinations of plasma histamine levels have been made under these conditions, but it is unlikely that they would show any increase, since Emmelin *et al.* (5) obtained detectable elevations of plasma histamine levels only with doses which produced marked physiological changes. They observed

that the plasma concentration had to be increased by about 25 per cent before it could be detected by Barsoum and Gaddum's method of extraction. Unless we assume an accumulation of the drug at or in the parietal cell until effective concentrations are obtained, it appears from these observations that if the histamine normally found in the plasma by investigators is in an active state, only a very slight increase in active plasma histamine concentration is necessary to activate the parietal cell. Present concepts of physiology and pharmacodynamics make this latter hypothesis unlikely. If physiological gastric secretion is due to an increase in active plasma histamine levels, we cannot expect to confirm this by the methods of histamine extraction presently in use. In other words, the parietal cell is far more sensitive to histamine than are the available methods for assaying histamine in blood.

A biological observation, namely, the incidence of headache in our experiments on humans, may be of significance. Those subjects who had headaches sometimes noticed them quite soon after a gastric response to histamine was observed. It is true that the headaches often occurred immediately after changing the dose as has been observed by Weiss *et al.* (9), but this was not always true. In five subjects headaches occurred after doses of histamine considerably less than maximal. We therefore ask this question: If the physiological response of the parietal cell is due to an increase in plasma histamine levels, why do we not frequently experience headache following the ingestion of food?

It has been suggested that histamine may play a physiological rôle not as a humoral agent but as a local chemical mediator of stimuli for gastric secretion. Emmelin and Kahlson (2) report parietal secretions resulting from a number of different stimuli; e.g., sham feeding or vagal stimulation, acetylcholine, gastric secretory phase, eserine, gastrin and the drug Priscol all contain an amount of active histamine. The presence of histamine in gastric juice has been confirmed by Hallenbeck and co-workers (22). They conclude that histamine may be the final link in the elaboration of parietal cell secretions. Consistent with this hypothesis are the findings that the histamine concentration in the stomach is high in the fundic portion (19), and histaminase is absent from the gastric mucosa. Emmelin and Kahlson suggest that histamine may be released in proximity to the parietal cell by gastrin, and that as it diffuses through this cell stimulation of secretion occurs. No explanation has been offered of the mechanism by which active histamine may be released from the plasma or tissues to exert its effects on the parietal cell. The evidence that histamine acts locally to stimulate gastric secretion during normal digestion is far from complete, and a conclusion of such a function must await more direct experimental proof.

In the final analysis, no direct proof exists for the participation of histamine in the normal mechanisms for gastric secretion, either as a humoral agent or as a local chemical mediator. On the other hand, neither of these possibilities is disproven by the available evidence.

SUMMARY

1. The histamine doses which produced minimal and maximal gastric secretory responses in the dog were established, and the threshold value was obtained in the

human. The minimal doses were similar in intact and in vagotomized total pouch dogs, whether histamine was administered by periodic subcutaneous or continuous intravenous injection. Maximal doses were also similar in pouch and intact dogs by either method of injection. These results indicate *a*) that vagotomy does not alter the gastric secretory response to histamine in the dog and *b*) that after subcutaneous injection no histamine is inactivated during its absorption from the subcutaneous tissues into the blood.

2. The maximal secretory response that could be elicited by histamine was very similar in intact dogs and in dogs with vagally denervated pouches of the entire stomach. It amounted, on the average, to 80 cc. per hour with a total acid concentration of 140 mEq. per liter.

3. Humans are much more sensitive to histamine than dogs; acid responses were obtained with an average dose of 0.004 μ g. base per kgm/min. or about one tenth of the dose necessary to produce a minimal response in the dog.

4. The parietal cell appears to be the most sensitive to histamine of any in the intact body, although it is possible that this sensitivity may be equalled at times by that of the cerebral vessels.

5. The characteristics of the gastric response to histamine, the possible mode of action by the drug to cause gastric secretion, and the evidence bearing upon the possible rôle of histamine as a physiological stimulant of gastric secretion are discussed.

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EFFECT OF D-AMPHETAMINE ON GASTRIC HUNGER CONTRACTIONS AND FOOD INTAKE IN THE DOG

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ONE theory of the mode of action of d-amphetamine in reducing food intake is that the drug depresses gastric hunger contractions and thereby reduces hunger sensations. This theory has recently been shown to be untenable because cutting of all the extrinsic nerves to the stomach, both sympathetic and parasympathetic, did not alter the depression of food intake produced by d-amphetamine (1). This showed that the action of the drug could not depend upon an alteration in the sensations arriving from the stomach.

Furthermore the evidence that d-amphetamine inhibits the gastric hunger contractions is inconclusive. Beyer and Meek (2) studied gastric motor activity in fasting dogs. They do not specify how many tests were made or how many dogs were observed, but they report that 30 mgm. of d-l amphetamine by stomach tube caused a preliminary increase in tone and motility 8 minutes after administration and a marked inhibition, even to cessation of motility some 40 minutes later. No other reports on the effect of amphetamine on the contractions of the empty stomach of the dog have been found. Kunstadter, Necheles and Weiner (3) studied gastric hunger motility in human subjects and concluded that significant inhibition of hunger contractions resulted from the introduction of 10 mgm. of d-l amphetamine sulfate into the stomach after a latent period of about 50 minutes. They present only one tracing in support of this statement and do not state how many tests were performed or what variability in response was encountered.

Studies on the effect of amphetamine on the rate of gastric evacuation are in substantial agreement, showing that amphetamine causes the stomach to empty more slowly than normal (1) which suggests that the drug depresses the motor activity of the filled stomach.

The present study was undertaken in order to determine the effect of small parenterally-administered doses of d-amphetamine upon the gastric hunger contractions and to correlate the effects upon gastric motility with the effects upon the amount of food ingested.

METHODS

Intact dogs. Two normal dogs which had been used extensively for other studies on gastric hunger contractions were used in these studies. The hunger contractions were recorded by means of a balloon inflated with a small amount of air (10 to 20 cc.) attached to a stomach tube which in turn was connected to a water manometer. The arm of the glass float of the manometer recorded upon a slowly moving kymograph drum. The dogs were trained to swallow the balloon and to lie quietly on the table without restraints during the recording of the contractions.

The diet of the dogs was standardized. To one box of dehydrated commercial

Received for publication January 26, 1948.

TABLE I. EFFECT OF SUBCUTANEOUSLY INJECTED D-AMPHETAMINE ON GASTRIC CONTRACTIONS AND FOOD INTAKE

EXPT. NO. ¹	SUBSTANCE INJECTED	DOSE	FOOD INTAKE	TYPE OF HUNGER CONTRACTIONS OBSERVED.	
				Control period	Change after injection
a. Intact dogs					
			grams		
1	Saline	1 cc.	456	II	No change
2	Saline	1 cc.	450	II	No change
3	Saline	1 cc.	453	II	Change to type III
4	Saline	1 cc.	451	II	No change
5	d-A ²	0.5 mgm.	77	I	Depressed for 25 min.
6	d-A ²	0.5 mgm.	31	II	Reduced to tonus rhythm for last 20 min.
7	d-A ²	1.0 mgm.	100	II	Depressed to tonus rhythm for 15 min.
8	d-A ²	1.0 mgm.	None	II	No change
9	d-A ²	1.5 mgm.	20	II	No change
10	d-A ²	1.5 mgm.	250	II	Change to type I for 45 min., then back to II
11	d-A ²	2.0 mgm.	None	II	Change to type I for 15 min.
12	d-A ²	2.0 mgm.	None	II	Change to type I for 15 min.
13	Saline	1 cc.	447	II	Change to type III
14	Saline	1 cc.	451	II	No change
15	Saline	1 cc.	443	II	No change
16	Saline	1 cc.	453	II	No change
17	d-A	0.5 mgm.	450	II	No change
18		0.5 mgm.	440	II	No change
19		1.0 mgm.	430	II	Reduced to type I for 10 min.
20		1.0 mgm.	None	II	No change
21		1.5 mgm.	None	II	No change
22		1.5 mgm.	None	II	Reduced to type I for 20 min.
23		2.0 mgm.	None	II	Reduced to tonus rhythm for 50 min.
24		2.0 mgm.	None	II	Reduced to tonus rhythm for 50 min.
b. Vagotomized total pouch dogs					
25	Saline	1 cc.	3	I	No change
26	Saline	1 cc.	3	I	Type II
27	Saline	1 cc.	3	I	No change
28	Saline	1 cc.	3	I	No change
29	Saline	1 cc.	3	I	Change to type II
30	d-A	5 mgm.	None	I	No change
31	d-A	5 mgm.	None	I	No change
32	d-A	10 mgm.	None	II	No change
33	d-A	10 mgm.	1 mouthful	I	No change
34	d-A	10 mgm.	None	I	No change
35	Saline	1 cc.	3	I	No change
36	Saline	1 cc.	3	I	No change
37	Saline	1 cc.	3	I	No change
38	Saline	1 cc.	3	I	No change
39	d-A	10 mgm.	None	I	No change
40	d-A	10 mgm.	2 mouthfuls	I	No change
41	d-A	10 mgm.	2 mouthfuls	I	No change
42	d-A	10 mgm.	None	I	No change

¹ Experiments 1 to 12, dog 1; 13 to 24, dog 2; 25 to 34, dog 3; 35 to 42, dog 4.² d-Amphetamine sulfate. ³ Animals ate well; exact amount was not measured.

dog food (Pard, Swift) was added 200 cc. of water. This mixture was offered to the dogs for 30 minutes each day at the same time.

One day each week a test was performed. Food was withheld for 24 hours preceding each test. The gastric motility of the fasting dog was recorded during a 30-minute control period. The substance to be tested on that day was then administered by subcutaneous injection and the recording was continued for one hour longer. The substances tested consisted of d-amphetamine sulfate in doses of 0.5, 1.0, 1.5 and 2.0 mgm. and 1 cc. of 0.9 per cent sodium chloride as a control. At the end of the one hour postinjection period of recording, the stomach tube and balloon were removed and the usual daily ration of food, as described above, was offered to the

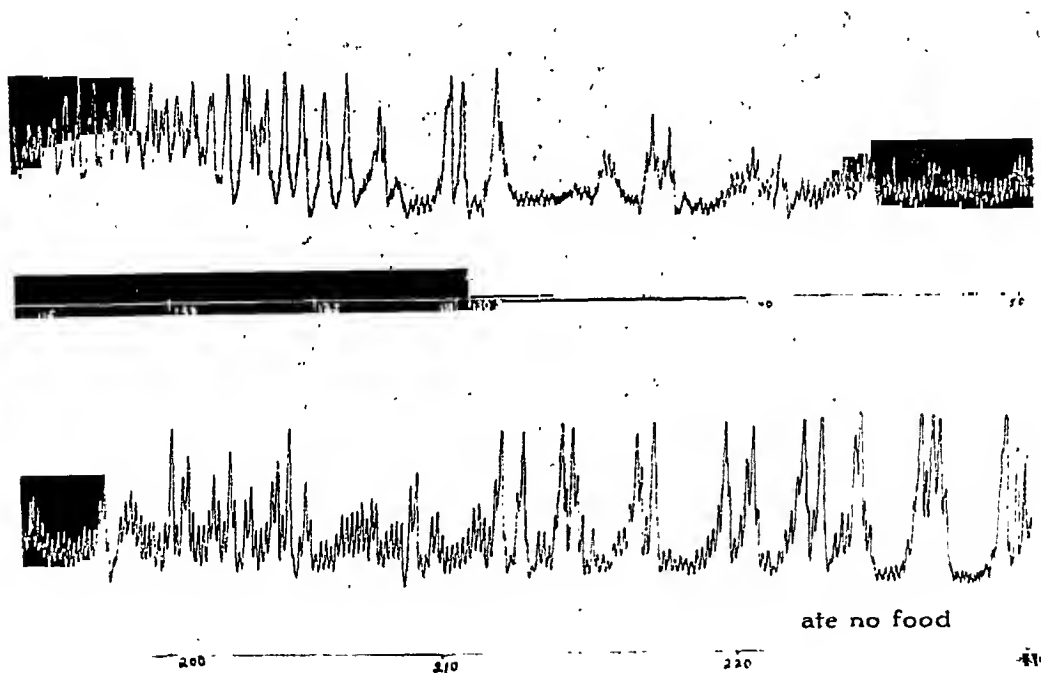


Fig. 1. KYMOGRAPHIC TRACING of gastric motility in an intact dog. At the arrow 2 mgm. d-amphetamine sulfate was injected subcutaneously. Contractions were depressed for a short period and then full recovery occurred. In spite of the return of contractions, the dog ate none of the food that was offered to it immediately after the tracing was discontinued.

dog for one hour. The amount of food eaten was determined by weighings before and after eating.

Dogs with vagotomized gastric pouches. Two dogs with pouches of the entire stomach and esophogoduodenostomies were also used in this study. The vagal fibers to the stomach had been severed at the time of the operation. The procedure used in these animals was essentially the same as that used in the intact dogs except that the recording balloon was introduced through the stoma of the pouch instead of through the mouth. The doses of d-amphetamine sulfate were larger than those used in the intact dogs, 5 and 10 mgm. doses being used. These larger doses were employed because preliminary experiments with the smaller doses had shown them to be ineffective.

RESULTS

A summary of the results appears in table 1.

Intact dogs. In four control experiments on each dog the subcutaneous injection of 1 cc. of physiological saline failed to produce any significant change in the pattern of gastric motility and the average food intake was very similar in the two dogs, being about 450 grams. In the 16 experiments in which the d-amphetamine sulfate was administered, depression of both hunger motility and food intake was noted in most of the tests and occurred consistently with the higher doses. However, there was very poor correlation between these two effects of the drug. For example, full recovery of the gastric motor activity always occurred by the end of the one hour post-injection period of recording, but this was never accompanied by a return of desire for food, that is, the food intake was significantly below normal and in some instances no food was taken, despite the presence of strong gastric hunger contractions (fig. 1). Furthermore, the food intake was depressed in some tests even when no alteration in gastric motility had been recorded during the hour preceding the offering of the food.

When depression of gastric motility occurred the latent period between the injection and the onset of decreased contractions varied from 5 to 10 minutes.

Dogs with vagotomized pouches. The injection of the saline solution under the skin during the control experiments had no significant effect upon the gastric contractions. The results produced by the subcutaneous injection of 5 or 10 mgm. of d-amphetamine sulfate were consistent in every experiment. No alteration in gastric motility occurred but a marked depression of food intake, usually to zero, was observed in every test.

DISCUSSION

The finding that amphetamine fails to depress gastric motility in the vagotomized gastric pouch indicates that the integrity of the vagus nerve is necessary for the depression of gastric motility by this drug. In this respect amphetamine resembles intestinal and urinary extracts, for these substances have also been shown to be capable of depressing gastric motility only in animals in which the vagal pathways to the stomach are intact (4). The exact nature of this inhibitory effect, aside from the fact that it requires the vagus nerves for its production, is not known. The substances that produce it probably act either directly or indirectly upon the vagal centers of the brain. Whether the inhibition is due to depression of the activity of vagal motor-excitatory fibers or to stimulation of vagal motor-inhibitory fibers is unknown.

The findings of this study confirm and extend those of Harris, Ivy, and Searle (1) demonstrating that the depression of food intake by amphetamine cannot be attributed to the action of the drug upon the stomach but is more likely due to a direct central action. The action of the drug upon the stomach may thus be looked upon as a result and not a cause of the effect upon the central nervous system.

The results of this investigation also conform to the new viewpoint of the rôle of the gastric hunger contractions in the total pattern of food-taking behavior that has arisen as the result of recent investigations (1, 5). The gastric hunger contractions

and the hunger pangs associated with them, though undoubtedly important and perhaps essential for the development of appetite in the newborn, can no longer be considered to be the most important element in the control of food-taking behavior in the conditioned animal. This newer concept emphasizes the importance of the central nervous system in the manifestations of hunger and appetite and relegates the gastric hunger contractions to a subsidiary rôle as one of the many factors which may exert a reinforcing and facilitating action upon the 'centers' of the conditioned animal.

SUMMARY

d-Amphetamine sulfate produced a transient depression or abolition of gastric hunger contractions in intact dogs when administered subcutaneously. This inhibition of gastric motility could not be the sole or most important factor in the depression of food intake caused by amphetamine because *a*) after full recovery of the gastric contractions, food intake is still much depressed, *b*) with small doses of d-amphetamine sulfate, significant depression of food intake may occur without any depression of gastric motor activity, and *c*) in animals with vagotomized pouches of the entire stomach, d-amphetamine sulfate causes no suppression of the gastric contractions, even when large doses are given, but the characteristic depression of food intake is still observed.

The implications of these findings for the general theory of hunger and appetite are discussed.

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EFFECT OF RESECTION OF MESENTERIC LYMPH NODES ON INTESTINAL FAT ABSORPTION IN THE DOG

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THE observations reported here were made to determine whether intestinal fat absorption may be impaired and intestinal fat excretion increased by experimental interruption of the mesenteric lymph drainage. It was believed that such experiments might yield data relevant to the question, recently discussed by Frazer (1), of the extent to which fat absorption may be a function of the portal as well as of the intestinal lymphatic circulation. In addition they might be expected to furnish information on the mechanism of lacteal obstruction with steatorrhea which has been described in patients with tuberculous or lymphomatous mesenteric lymphadenitis (2-5).

EXPERIMENTS

In 10 mongrel dogs the absorption of fat was studied before and after resection of the mesenteric lymph nodes. In one control dog, fat absorption was studied before and after a sham abdominal operation. In this species, all or nearly all the mesenteric lymphoid tissue is localized in a single node or group of nodes in the root of the mesentery. Resection of this lymphoid mass, into which all the grossly visible lymphatics of the small bowel appear to converge, offers a convenient means of at least temporarily interrupting the mesenteric lymph flow.

In all experiments, the quantity of fat excreted in the feces was compared with dietary fat intake. In 5 animals, including the control, fecal and dietary nitrogen were also compared in order to obtain an index of non-specific variations in intestinal absorption involving both fat and protein which might result from operative manipulation or disturbed intestinal motility. In the same 5 animals, alimentary lipemia was studied by means of fat tolerance curves plotted from plasma lipid levels in the fasting state and at intervals after introduction of standard quantities of fat into the alimentary tract.

METHODS

Diet. A standard kennel ration (Dehydrated Pard, Swift & Co.) was supplemented with lard to provide a total fat content of 20 per cent of dry weight and a caloric value of 70 Calories per kilogram of body weight.

Chemical methods. Fat content of three-day pooled collections of feces was determined by Boutwell's (6) modification of Fowweather's (7) method in *experiments 1 to 6*, and by that of Fowweather and Anderson (8) in *experiments 7 to 10* and the control, *number 11*. Fecal nitrogen was determined by Woodman's (9) modification of the macro Kjeldahl method. Plasma lipid determinations were made by

Received for publication December 29, 1947.

Bloor's (10) method. For study of alimentary lipemia, plasma samples were obtained after an overnight fast, then at 2-, 4- and 6-hour intervals after administration of 100 grams of cottonseed oil by stomach tube.

Operative technic. Anatomically, the operation was based on the preliminary dissection of 6 dogs, in some of which the lacteals had been visualized by the antemortem administration of a fatty meal. The lacteals originate in a fine network beneath the serosa of the small bowel and converge to form larger collecting channels. These enter the mesenteric nodes which consist of several closely grouped or confluent masses of lymphatic tissue lying between the peritoneal layers in the root of the mesentery near the convergence of the mesenteric veins. Occasional smaller mesenteric lymph nodes, apparently unrelated to the lymph drainage of the small bowel, have been observed with considerable variability in other locations. These may occur along the lesser curvature of the stomach or near the pylorus, along the course of splenic or portal veins, or in the mesocolon near the larger branches of the inferior mesenteric vessels. Our findings agree with those of Bradley and Graham (11).

The operations were performed under drop ether anesthesia, with aseptic technic, two to four hours after administration of 67 to 100 grams of cottonseed oil. All the lymphoid tissue in the mesentery of the small bowel was removed by dissection with gauze and fingers. The entire small bowel was then explored and any remaining intact lymphatic vessels broken. Smaller nodes, unassociated with lymph drainage of the small bowel, were removed only when readily accessible.

A control operation in *experiment 11* consisted of celiotomy and manipulation of the viscera without interruption of the lymphatics.

Second operations consisted of re-exploration and destruction of newly formed mesenteric lymph channels in *experiments 3* and *4*, and of transthoracic ligation of the thoracic duct at the level of the aortic arch in *experiment 9*.

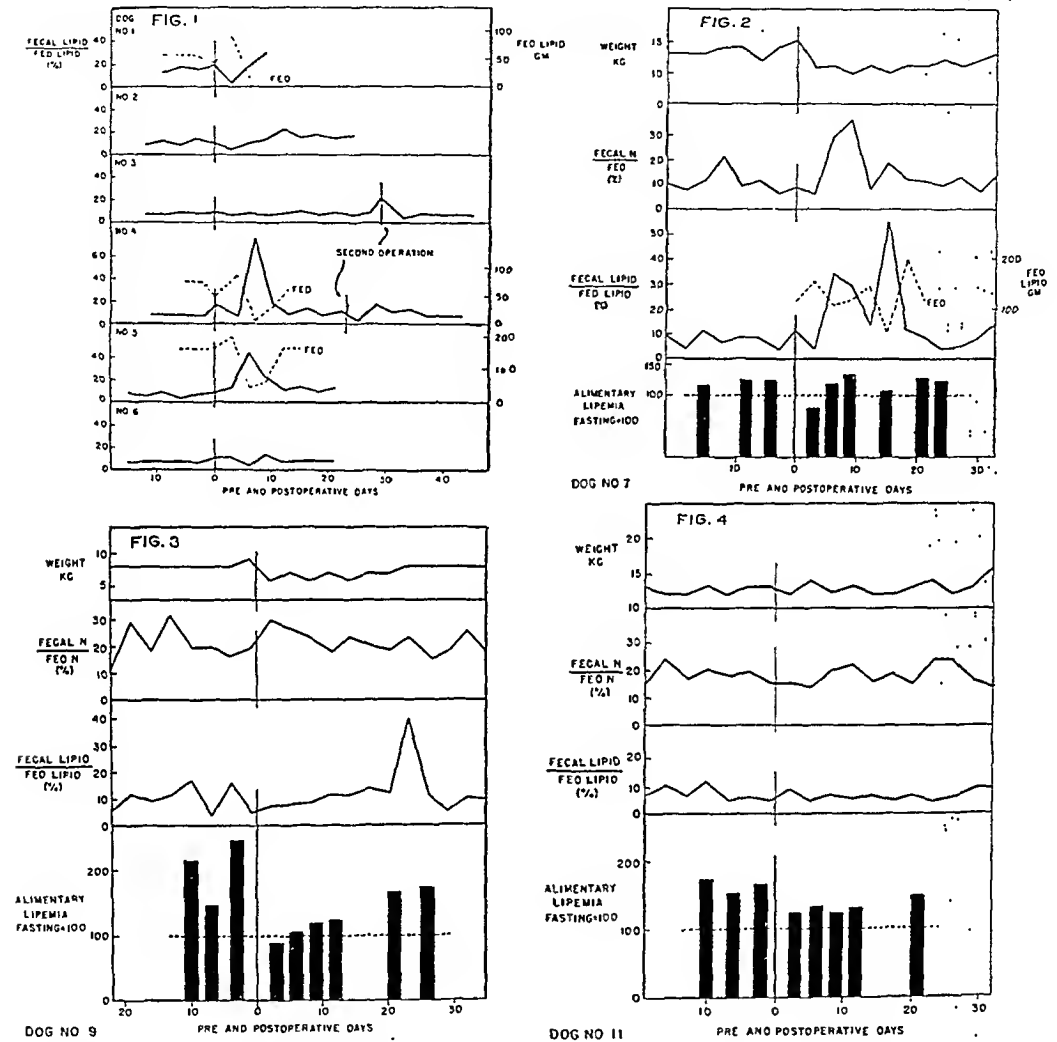
RESULTS

Effect of the operation on the fecal excretion of fat. Data on the fecal excretion of fat were obtained before and after the destruction of the lymphatic drainage of the small bowel in 10 dogs. The data for each of the first 6 dogs are graphed in figure 1. Reference to the figure will show that the operation was followed by an increase in the ratio of fecal to fed fat in only 3 of the 6 dogs, and namely, *dogs 1, 4* and *5*. The operation, however, simultaneously decreased the appetite and fat intake (see broken line in graphs). This indicated that the alteration of the ratio was due to the diminution of fat intake. Though during the period of diminished fat intake the endogenous fat excretion by the intestine as calculated from Sperry's data (12) was inadequate to account for the entire lipid output, the significance of these transient changes is open to question.

When the results on the first 6 dogs were at hand, it was decided to ascertain to what extent the operation also disturbed the ratio of fecal nitrogen output to dietary nitrogen intake. This was significantly altered in only one (*dog 7*) of the 5 dogs (*dogs 7* to *11*) studied (fig. 2).

Effect of the operation on the extent of alimentary lipemia. This point was studied because at the second operation on *dogs 3* and *4* it was found that though many lac-

teals in the mesentery had regenerated they were not as numerous as before the operation. Regeneration of lackals was observed at autopsy in all animals. In 6 it was also found at autopsy that small new mesenteric lymph nodes had formed at the site of previous extirpation. It was therefore thought that although the operation did not unequivocally influence the total absorption of fat, it might retard the



Figs. 1-4. FECAL FAT EXCRETION and alimentary lipemia before and after resection of mesenteric lymph nodes.

rate of absorption. This possibility was investigated by study of the alimentary lipemia curve after the administration of a fatty meal.

The results are shown in the graphs for dogs 7, 9 and 11 (figs. 2, 3, 4). The height of the bars indicates the highest level of plasma lipid at 2, 4 or 6 hours after the administration of the fatty meal, the overnight fasting level being represented as 100. In dogs 7, 8 and 9, no increase in plasma lipid occurred when the fat was fed on the third postoperative day. By the sixth postoperative day, normal increase in

plasma lipid was apparent after the fatty meal in *dogs 7* and *8*. This did not occur until after the twelfth postoperative day in *dog 9*. *Dog 10* was not affected by the operation and the control animal, *dog 11*, only very slightly affected.

The thoracic duct was ligated in *dog 9*, 62 days after resection of the mesenteric lymph nodes. Six days after thoracic duct ligation the alimentary lipemia curve was normal. At autopsy later, a large chyle-filled collateral channel was discovered which had not been present at the time of ligation of the main duct.

SUMMARY

Observations were made to determine whether intestinal fat absorption might be impaired or fat excretion increased by experimental interruption of the mesenteric lymph drainage.

Resection of the mesenteric lymph nodes in 10 dogs did not alter fecal fat and nitrogen excretion. In each, there was rapid re-establishment of anatomic and functional continuity of the interrupted mesenteric lymphatics. In 6 animals, partial regeneration of the nodes occurred. Alimentary lipemia was studied in 4 animals, 3 of which showed normal values six to twelve days after operation.

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GASTRIC ABSORPTION OF ETHYL ALCOHOL IN THE RAT

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ALTHOUGH the gastric absorption of ethyl alcohol was reported as early as 1847 (1), not until 1881, when Tappeiner (2) published the results of his investigations, were unequivocal experimental data presented showing not only that the stomach is permeable to alcohol, but also that the extent of absorption may be appreciable. During the next 25 years the work of Tappeiner (2) was confirmed by others (2-11).

Berggren and Goldberg (12) assumed, from investigations on human subjects and on cats, that the passage of alcohol through the gastric mucosa is purely a process of diffusion and that, therefore, the blood alcohol level will rise higher and more rapidly the greater the concentration of the ingested alcohol. The data of Haggard *et al.* (13) tend to confirm this. A contrary conclusion was reached by Delhougne (14) who failed to observe marked gastric absorption of alcohol in dogs. Subsequently, data similar to those of Delhougne were reported for rats by Rasmussen (15) and Dybing and Rasmussen (16), but the latter attributed their findings to the anatomy of the rat stomach. Appreciable absorption of both methyl and ethyl alcohol from the rat stomach was reported by Haggard, Greenberg and Lolli (13).

To investigate further the questions of absorption from the stomach of the rat, the effect of hydrostatic pressure on absorption and diffusion through the gastric walls, the following studies were undertaken: *a*), effect of ligating both cardia and pylorus on hydrostatic or 'filtration' pressure; *b*), diffusion of ethyl alcohol through the ligated, excised stomach and through the ligated nonexcised stomach; *c*), effect of gastric mucosa and stomach walls on the metabolism of ethyl alcohol; *d*), effect of protein-binding by the blood and by the stomach tissue on recovery of alcohol; *e*), excretion of alcohol through the respiratory pathway; and *f*), portal and right ventricular blood levels of alcohol as indications of gastric absorption and distribution in the body water.

METHODS

The animals used were Wistar, albino male rats weighing approximately 170 to 225 grams. Prior to the experiment these animals were fasted for 18 to 20 hours but were allowed water *ad libitum*. Anesthesia was induced by the intraperitoneal injection of 40 mgm. of pentobarbital sodium per kgm. body weight. Alcohol was injected into the stomach near the cardia with a no. 27 needle. To obviate expulsion of alcohol at the puncture site, the stomach at this point was gently pinched with a hemostat as the needle was withdrawn and for one or two seconds thereafter. Following the introduction into the stomach of 1.0 ml. of a 5 M (23.8 per cent by weight) solution of ethyl alcohol per 200 grams of body weight, the alcohol was allowed to

Received for publication March 25, 1948.

remain in the stomach for exactly 20 minutes, at the end of which time the stomach was excised, emptied of its unabsorbed alcohol and minced by grinding. Just prior to excision a portal vein and/or a right ventricular sample of blood was obtained. Quantitative determinations of alcohol unabsorbed from the stomach, alcohol in the blood and stomach tissues and, in a number of instances, respired and diffused alcohol were then made by the methods outlined below.

All the alcohol injected into the stomach of the rat may be accounted for on the basis of the observations of Harger *et al.* (19) who found no changes in the concentration relations of ethyl alcohol in blood, brain and liver of dogs sacrificed at time intervals varying from 15 minutes to 12 hours after administration of alcohol. They also found alcohol to be stored in about the same proportion as the water content of the materials analyzed. For muscle tissue, the alcohol was approximately 0.90 of that stored in blood, and average ratios for brain, liver and intestine were close to this figure.

In our study, it was assumed that the speed with which various parts of the body reach equilibrium in the storage of absorbed ethyl alcohol was at least as rapid in the rat as in the dog and that the ventricular levels of alcohol were representative of that distributed in the body water after correction by the factor of 0.90 cited above. On the basis of the observations of Wynn and Haldi (20) on the water content of the albino rat on a normal laboratory diet, the water content of the animals used in this investigation was considered to be 62 per cent of the total body weight.

Ethyl alcohol in blood, in stomach contents, in stomach tissues, peritoneal fluid and respired air was determined by the procedures described by Widmark (17) and by Nicloux (18). The determinations were nonspecific, depending on the oxidation of the alcohol to acetic acid by an excess of $K_2Cr_2O_7$ and H_2SO_4 and iodometric titration of unreacted $K_2Cr_2O_7$.

To minimize loss of alcohol by vaporization, 2 ml. blood samples were collected in a cold syringe and further chilled by placing in a test tube immersed in an ice bath. Following the cooling and mixing of the blood, one ml. was placed into the cup of each of two flasks, similar to those used by Widmark (17), containing a measured amount of the $K_2Cr_2O_7$ - H_2SO_4 oxidizing mixture. After two hours at 85°C. the diffusion of the alcohol from the cup into the dichromate oxidizing mixture was found to be complete. Determinations were made in duplicate. Blank determinations with one ml. of blood drawn from rats injected intragastrically with normal saline in a volume equal to the alcohol which would have been given were equivalent to 0.05 mgm. of ethyl alcohol. The reduction of the dichromate-sulfuric acid oxidizing mixture itself was found to be equivalent to 0.06 mgm. of alcohol. The additive saline and dichromate-sulfuric acid oxidizing mixture blanks were equivalent to 0.11 mgm.

The determination of residual alcohol in the stomach cavity was made by excising and transferring the stomach to a 50 ml. beaker containing a volume of iced, distilled water sufficient to cover the organ, cutting the stomach and washing it under the water. Two additional washings of the stomach were then made in separate beakers, following which all three washes were quantitatively transferred to a 100 ml. volumetric flask and diluted to the mark, the alcohol so obtained being directly distilled, subsequent to the addition of protein precipitant (saturated aqueous picric acid solution) according to the method of Nicloux (18). An aliquot of the distillate was placed in a digestion tube containing $K_2Cr_2O_7$ and H_2SO_4 (6N). After remaining in the digestion tube at 85°C. for one hour, the alcohol was determined iodo-

metrically. Peritoneal washings obtained in some of the experiments to assess qualitatively the extent of diffusion through the gastric walls in the closed abdomen were treated like blood in that alcohol recoveries were obtained from aliquots placed in the cups of Widmark flasks.

Subsequent to the third washing the stomach was rendered brittle by contact with dry ice and pounded into very small fragments in a cold steel mortar. The fragments were then placed in a glass grinding tube containing exactly 5 ml. of distilled water and ground while the tube was kept immersed in ice water. Next, the cold supernatant liquid was centrifuged for five minutes in a stoppered tube and immediately chilled. A 0.5 ml. aliquot was then analyzed for alcohol by the Widmark procedure.

To minimize the loss of alcohol from presumed intestinal diffusion when the stomach was permitted to remain in the open abdomen as described below in (c) and (d), ca. $\frac{3}{4}$ ", midline incisions were made almost parallel to the stomach. Ligations of cardia and pylorus were made with care to exclude major blood vessels from the ligatures. To avoid leakage from the ligated stomach, two ligatures with number 40 cotton sutures, one immediately behind the other, were applied to both the cardia and pylorus. Ten checks for leakage, made by inserting cotton pledgets in loops of oesophagus and duodenum immediately adjoining the cardiac and pyloric ligatures, respectively, were negative by the Widmark method.

The effect of 'filtration' or hydrostatic pressure was determined by comparing the absorption and the ventricular blood levels of alcohol obtained under the following conditions. *a.* Stomach ligated at cardia and pylorus and replaced in the abdomen which was then closed by suturing. *b.* Stomach ligated at cardia and cannulated at pylorus, other conditions being the same as in *a.* *c.* Same as in *a* except that abdominal incision was not closed. *d.* Same as in *b* except that abdomen was not closed. *e.* Stomach doubly ligated and exteriorized on sutured abdomen. *f.* Same as in *e* but cannulated at pylorus.

The cannula used (devised by Mr. J. Lowen, fig. 1) was sealed with physiological saline to prevent loss of alcohol by vaporization.

After measuring the *in vitro* diffusion of alcohol from excised ligated stomachs (table 2) immersed in physiological saline at 37.5°C. for 20 minutes, *in vivo* diffusion was measured by three methods: *a)* exteriorizing the ligated stomach on the sutured abdomen and collecting the diffused alcohol through an aeration chamber into which the rat was placed for 15 minutes; *b)* suspending the whole stomach in normal saline at body temperature by means of a saline-filled container placed firmly against the body of the animal in the prone position; and *c)* introducing saline into the closed abdomen for a 20-minute period at the end of which time the alcohol in the peritoneal cavity was determined as previously described. These washings were compared with similar washings in animals injected intravenously with a sufficient quantity of alcohol to yield blood levels equal to the mean of those obtained with the intragastric injection of alcohol, the purpose of the latter procedure being to account for diffusion of alcohol into the peritoneal cavity by way of the intestines or routes other than the stomach.

The aeration chamber was a cylinder 10½" long and with a diameter of 3½". Into this chamber maintained at 36°–38°, air, first filtered through cotton wadding

and charcoal, then bubbled through hot acid dichromate to remove reducing substances and, finally, humidified by bubbling through two tubes in series, containing warm water, was admitted by suction at a flow rate of 0.3 liter per minute. The air which passed over the exposed stomach, kept moist by a suitably sized layer of cheesecloth dampened with saline, was collected in a bubbler holding the dichromate-sulfuric acid oxidizing mixture. The bubbler itself was immersed, during the collection of chamber air, in a boiling water bath colored with a bone-black suspension to prevent light from affecting the mixture.

Since it was not feasible to draw blood samples directly from the gastric veins, blood drawn for the purpose of qualitatively assessing gastric alcohol absorption was obtained from the portal vein as close to the stomach as possible in heparinized

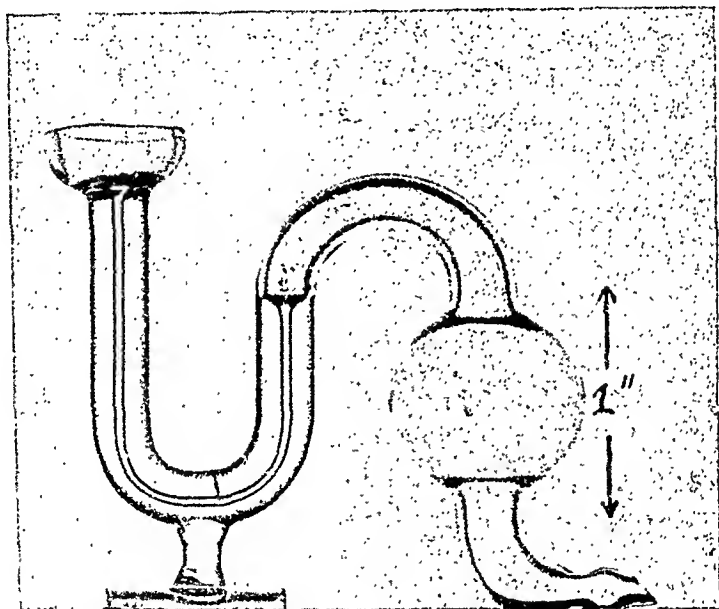


Fig. 1. APPARATUS for cannulating the rat stomach.

syringes. The approximate amount of alcohol distributed in the body water was calculated from right ventricular blood samples.

The respiratory excretion of alcohol was measured by means of the aeration chamber already described.

The effect of gastric mucosa and of stomach wall minus the mucosa on the metabolism and binding of alcohol was determined by conducting recovery experiments on *a*) excised, ligated stomachs injected with alcohol and incubated for 20 minutes (table 2), *b*) on gastric mucosa itself (table 1b), and *c*) on minced stomach walls minus the mucosa (table 1b).

Appropriate control determinations were made in all experiments.

RESULTS

Data relative to the percentage recovery of ethyl alcohol from *in vitro* experiments with the dichromate oxidizing mixture itself, with blood, with the aeration

chamber and with stomach tissues are given in table 1. Results of the experiments designed to yield quantitative data on gastric absorption and ventricular blood levels in both cannulated and noncannulated animals, with the stomachs in the several positions described earlier, are given in table 3. The latter data suggest that hydrostatic pressure does affect absorption; moreover, from the *in vitro* and the *in vivo* data, it is obvious that ethyl alcohol can diffuse through the stomach walls into the peritoneal cavity.

That gastric mucosa may metabolize alcohol is suggested by experiments performed with activated and deactivated (by heating to 60°C. for 30 minutes) gastric mucosa to determine the extent of metabolic conversion and protein-binding of

TABLE 1. RECOVERY OF ETHYL ALCOHOL

KIND OF SAMPLE	NO. OF SAMPLES	ALCOHOL ADDED	ALCOHOL RECOVERED
<i>a. Twenty minutes after addition of the alcohol</i>			
		<i>mgm./ml.</i>	<i>% ± σ_M</i>
Dichromate-H ₂ SO ₄	11	1.13	100.4 ± 5.9
Venous blood.....	11	1.13	100.1 ± 1.8
Aeration chamber.....	8	1.13	101.3 ± 1.8
<i>b. After 20 minutes incubation at 37.5° C. with stomach tissue</i>			
Stomach ¹ , activated.....	Composite	1.13	93.7
Stomach ¹ , deactivated ³	Composite	1.13	92.7
Mucosa ² , activated.....	8	0.64	83.5 ± 4.3
Mucosa ² , deactivated ³	8	0.64	89.5 ± 1.9

¹ After removal of mucosa, the stomachs of 20 rats were minced in a Waring blender with 100 ml. of phosphate buffer and cracked ice. Four 5 ml. aliquots of a total volume of 185 ml. of homogenate were incubated with 5 ml. of ethyl alcohol solution equivalent to 11.30 mgm. of alcohol, and then analyzed.

² The gastric mucosa of each of 8 rats was separately mixed with 15 ml. of phosphate buffer. Five ml. portions of active tissue were incubated with 4 ml. of ethyl alcohol solution equivalent to 4.52 mgm. of alcohol and aliquots were taken for analysis.

³ Five ml. aliquots of buffered tissues were deactivated by heating to 60°C. for 30 minutes, then treated identically as active preparations.

alcohol (table 1b). As regards the gastric walls minus mucosa, results (table 1b) indicative of metabolic effects were negative but it is likely that protein-binding and perhaps adsorption accounted for an approximately 7 per cent loss (non-recoverable alcohol). Since, however, the alcohol must first be in intimate contact with these tissues to be metabolized, bound or adsorbed, it is presumed to have first been absorbed.

Data obtained regarding respiratory excretion, using 10 rats, gave a mean value of 0.2 ± 0.02 mgm. per animal during the first 15 minutes after injection of the alcohol into the stomach.

Diffusion experiments by use of the aeration chamber on rats with stomachs exteriorized gave a mean value of 0.5 ± 0.04 mgm. per animal over a 15-minute period contrasted to 0.3 mgm. for rats whose stomachs were lying *in situ* in the open abdominal cavity. The latter figure, however, was obtained on only 4 animals, compared to 10 rats used in obtaining the former. When attempts to determine

diffusion by the peritoneal washing technique were made, five rats with stomachs enclosed in the sutured abdomen after alcohol injection had an average of only 0.3 mgm. of alcohol diffusing from the stomach into the peritoneal cavity, a value which is in fair agreement with that found in the aeration procedure. However, the

TABLE 2. RECOVERY OF ALCOHOL FROM 9 LIGATED, EXCISED STOMACHS IMMERSSED IN PHYSIOLOGICAL SALINE AT 37.5°C. FOR 20 MINUTES

	AMOUNT INJECTED	AMOUNTS RECOVERED IN			
		Diffusate	Contents	Tissue	Total $\pm \sigma_M$
Milligrams.....	223	6.8	187.0	12.6	206.4 ± 3.8
Per cent.....	100	3.0	83.8	5.6	92.4 ± 0.8

TABLE 3. GASTRIC ABSORPTION AND BLOOD LEVELS OF ETHYL ALCOHOL IN RATS 20 MINUTES AFTER THE INTRAGASTRIC INJECTION OF 1.13 GRAMS OF ALCOHOL PER KGM. BODY WEIGHT

STOMACH POSITION	WEIGHT OF RATS	NO. OF RATS	ETOH DISAPPEARING FROM STOMACH	NO. OF RATS	VENTRICULAR BLOOD LEVELS
<i>a. Stomach ligated at both cardia and pylorus</i>					
Enclosed in abdomen	gm. 197 (186-228)	7	% $\pm \sigma_M$ 41.7 ± 2.1 (33.3-49.2)	7	mgm. % $\pm \sigma_M$ 48 ± 4.3 (33-64)
<i>In situ</i> in open abdomen	198 (174-215)	9	38.0 ± 2.0 (28.6-46.2)	14	47 ± 5.6 (20-85)
Exteriorized on sutured abdomen	201 (188-227)	9	38.6 ± 4.7 (17.3-64.5)	9	41 ± 5.9 (15-64)
<i>b. Stomach ligated at cardia and cannulated at pylorus</i>					
Enclosed in abdomen	194 (176-218)	15	41.9 ± 4.3 (14.1-83.5)	14	46 ± 8.0 (5-96)
<i>In situ</i> in open abdomen	189 (165-220)	10	22.9 ± 4.6 (5.7-42.8)	10	33 ± 9.3 (6-102)
Exteriorized on sutured abdomen	201 (190-219)	7	24.6 ± 4.3 (12.3-45.4)	9	29 ± 6.9 (9-71)

average diffusion value in four rats, the stomachs of which were suspended in saline, was considerably higher, being 0.9 mgm. It will be noted (table 3) that none of these diffusion values is great enough to account for marked differences in absorption between the stomachs in the several positions. In fact, even though diffusion determined with 10 excised stomachs reached a value of 3 per cent in 15 minutes (= to ca. 6.8 mgm. per 200 gram rat), again this difference is not great enough to account for that between the stomachs in the various positions.

As would be expected, portal blood levels were greater than ventricular levels in all animals in which simultaneous samples of both types of blood were taken. For

example, in five typical but random cases, the values for portal blood were 88, 79, 77, 69 and 66 mgm. per cent, while the corresponding ventricular levels were 33, 47, 62, 41 and 39 mgm. per cent. The consistently high portal levels at points very close to the stomach were indicative of marked absorptive activity by the stomach, a finding fortified by the positive evidence of diffusion, though small, through the gastric walls. High ventricular blood levels, 80.3 and 85.4 mgm. per cent, were found in two animals with ligated but not cannulated stomachs enclosed in the abdomen. The validity of these blood samples is questioned since the animals were *in extremis* at the time the blood was collected. Data on these two animals therefore are not included in table 3, inasmuch as the calculated values for metabolized alcohol are negative.

DISCUSSION

The currently reported data confirm the observations of Haggard *et al.* (13) regarding the gastric absorption or disappearance of ethyl alcohol in the rat and extend the assumptions of Berggren and Goldberg (12) relative to the diffusion of alcohol in the stomach. While it was the opinion of the latter authors that the absorption of alcohol is purely the result of a process of diffusion into the gastric mucosa, it has not, to our knowledge, been previously demonstrated that *in vivo* alcohol may diffuse into the highly absorptive peritoneal cavity. This suggests that when volatile substances or gases, like CO₂, are introduced into the stomach, the peritoneum may function as an absorptive organ in a sense not hitherto fully recognized. Furthermore, it is likely that such a function is even more important in the absorption of such substances from the intestines.

The rate of disappearance of ethyl alcohol from the stomach of the rat is so variable that a very large series of animals would be necessary to demonstrate statistically significant differences between the rates of absorption in different stomach positions or between cannulated and noncannulated stomachs.

Nevertheless an analysis of the data in table 3 indicates certain consistencies: *a*) in five of the six groups the mean values for absorption of alcohol were lower in the cannulated than in the noncannulated animals; *b*) the lowest blood levels found were also in the cannulated groups in each of the six categories; *c*) while the mean highest absorption and blood levels were associated with both cannulated and noncannulated stomachs enclosed in the abdomen, the higher levels occurred with greater regularity in the case of the doubly ligated stomachs as evidenced by the standard deviations of the respective values. The data then strongly suggest that there are real differences in absorption resulting from hydrostatic pressure.

Since the pH values (2.1 to 3.4) of a control series of 10 stomachs were not particularly variable, it is felt that differences in gastric acidity could not have been a cause of the individual variation in absorption. It was presumed, however, that the size of the stomachs, which were so variable that the range of the ratio of the body weight to the moist weight of 22 stomachs was 109.8:1 to 202.4:1, might explain the noted inconsistency in gastric absorption among identically manipulated stomachs. As the stomachs in experimental animals could not be weighed without excision, and as such stomachs had to be minced quickly to avoid loss of alcohol, resort was had to approximate volume determinations of the preinjected, collapsed stomachs by

measurements of length, width, and thickness. Unfortunately, here, too, no correlation was observed between degree of absorption and external stomach dimensions, indicating that other factors, perhaps the state of the mucosa or walls, were operative in causing the variability.

Originally, since cannulated stomachs were not subjected to the factor of undue and variable hydrostatic pressure, it was anticipated that results among animals of a given group would be much more uniform in the cannulated than in the noncannulated. That this did not prove to be so again points to factors other than the pH, weight or volume dimensions of the stomach.

Seven animals with ligated but not cannulated stomachs enclosed in the abdomen (table 3) absorbed from 33.0 to 49.2 per cent of the alcohol injected with a mean absorption of the 41.7 per cent. The right ventricular blood alcohol levels ranged from 33.4 to 64.0 mgm. per cent with a mean of 48 mgm. per cent.

If it is assumed that a state of equilibrium exists between ventricular blood and body water at the end of 20 minutes, the alcohol in the body water of these seven animals may be approximated from the right ventricular blood levels (19) on the basis that 62 per cent of the total body weight is attributable to body water (20) and that 80 per cent of the blood by volume represents water (22). The difference between total alcohol absorbed and that estimated to be in the body water is considered to have been metabolized. For the seven animals noted above, the average is 14.4 milligrams per 100 grams of body weight at the end of the 20 minutes. This value for metabolized alcohol is not too distant from the figure of 11.4 milligrams per 100 grams of body weight obtained from the data of Le Breton (21) on the metabolism of alcohol by Wistar rats.

SUMMARY

1. Hydrostatic or 'filtration' pressure apparently affects the absorption of ethyl alcohol from the stomachs of rats. Ethyl alcohol may diffuse through the walls of the nonexcised stomach and be absorbed by the peritoneum. The mean percentage of alcohol absorbed in 20 minutes from stomachs ligated at both cardia and pylorus and injected with one ml. of 23.8 per cent by weight (5 M) alcohol per 200 grams of body weight was as follows: *a*) stomach enclosed in abdomen, 41.7; *b*) *in situ* in open abdomen, 38.0; and *c*) exteriorized on the sutured abdomen, 38.6. In the cannulated animals, the respective values were 41.9, 22.9 and 24.6. Mean ventricular blood levels for the corresponding series of observations were, in mg. per cent, 48, 47 and 41 for the noncannulated and 46, 33 and 29 for the cannulated.

2. That gastric mucosa may metabolize alcohol is suggested by experiments performed with activated and deactivated mucosa. Evidence of metabolism of alcohol by the gastric walls minus mucosa was entirely lacking, but protein-binding and possible adsorption accounted for about 7 per cent of nonrecoverable alcohol.

3. Presumptive evidence indicates that under the conditions of these experiments, about 14.4 mgm. of ethyl alcohol per 100 grams of body weight may be metabolized by the Wistar rat in 20 minutes.

The authors acknowledge gratefully the assistance of Miss Josephine Schaffer and especially that of Mrs. Jane U. Harrison in the conduct of these experiments.

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ABSORPTION OF BLOOD FROM THE PERITONEAL CAVITY OF THE DOG¹

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STUDIES dealing with the changes of the plasma volume and the plasma protein concentration which occur following a single nonfatal hemorrhage in normal dogs (1-9) have revealed that there is a prompt but gradual return of fluid and plasma protein to the circulation. The initial increase of plasma volume is apparently the result of the addition of fluid relatively poor in protein. Consequently, while the plasma volume may be found to equal or exceed the control level in from several hours to some 70 hours, the restoration of the plasma protein (albumin) may still be incomplete at the end of several days. Further, the re-expansion of the plasma volume occurs without early replacement of the erythrocytes. In fact, observations which were made in from one to four days following hemorrhage revealed an apparent loss in red cells from the circulation (10).

In some preliminary observations (11) it was found that absorption takes place quite promptly when a large amount of a dog's own blood is placed into the peritoneal cavity. In these experiments 25 per cent of the measured blood volume was transferred directly from the femoral artery into the peritoneal cavity, and only a small amount of bloody fluid could be detected when laparotomy was performed 24, 48 and 96 hours after treatment of the animals in this manner. That intact red cells are absorbed promptly following their intraperitoneal injection into normal dogs has been shown recently by means of red cells labeled with radio-iron incorporated in the hemoglobin (12). It has also been found that both hemoglobin and blood plasma when given intraperitoneally to a protein-fasting dog are used effectively to supply the protein requirements of the animal (13, 14).

In the present study normal dogs and dogs made hypoproteinemic by diet were subjected to a single nonfatal hemorrhage, and, in certain of the animals, the withdrawn blood was transferred directly into the peritoneal cavity. These experiments were undertaken to determine whether the erythrocytes and plasma proteins absorbed from the peritoneal cavity contribute measurably to the restoration of these components in the circulation following an acute blood loss.

Received for publication March 5, 1948.

¹ Aided by a grant from Wyeth Incorporated, Philadelphia. A preliminary report of this work was presented before the American Society of Biological Chemists, Chicago, Illinois, May 18, 1947 (Federation Proc. 6: 288, 1947).

METHODS

Young adult dogs were used in this study and were placed on a constant diet² for at least two weeks before the experiments were undertaken. Food was then withheld for 18 to 24 hours, and determinations of the plasma volume, hematocrit, hemoglobin concentration and concentrations of the plasma proteins were carried out. The determinations were repeated one week later, and if the plasma volume figures agreed with the first set the experiment was continued. The animal was then placed under sodium pentobarbital³ anesthesia and 25 per cent of the measured blood volume was withdrawn by direct puncture of the femoral artery. In one group of animals, serving as controls, the blood was collected in a suitable container. In a second group of animals the blood withdrawn from the femoral artery was injected immediately into the peritoneal cavity, employing suitable aseptic precautions. The bleeding time in the experiments reported never exceeded 15 minutes. Observations on the blood were then repeated 24 hours after the hemorrhage, during which period neither food nor water was allowed, and again 96 hours following the hemorrhage. The constant prehemorrhage diet was reinstituted upon completion of the 24-hour observations.

In addition to the experiments which were carried out on dogs receiving the basal ration, the same procedures of bleeding and of bleeding and injection into the peritoneal cavity of the withdrawn blood were performed on animals made hypoproteine-mic by diet. In the latter case the dogs were placed on a low protein diet⁴ for a period of three weeks before the experiments were undertaken.

The blood determinations were carried out on venous blood withdrawn from the jugular vein with minimal stasis. The blood was heparinized and sampled immediately for the determination of the hematocrit and the hemoglobin concentration. The hematocrit value was determined, in duplicate and undiluted, with the aid of Van Allen hematocrit tubes, carrying out the centrifugation at 3000 r.p.m. for one hour. The hemoglobin concentration was determined by the method of Van Bruggen (15) adapted to the Evelyn photoelectric colorimeter. The plasma volume was determined by the direct method of Gregersen and Stewart (16) as adapted to the photoelectric colorimeter by Gibson and Evelyn (17). The total plasma protein and albumin concentrations were determined by the microKjeldahl procedure; centrifugation was employed, as suggested by Robinson, Price and Hogden (18), for globulin separation after precipitation with 22.2 per cent sodium sulfate solution.

The whole blood and red cell volumes were calculated from the determined plasma volume and hematocrit values from the following relations:

$$\text{blood volume} = \frac{\text{plasma volume} \times 100}{100 - \text{hematocrit}} \text{ and, red cell volume} = \text{blood volume} - \text{plasma volume}.$$

² The basal diet consisted of: casein, 20 per cent; glucose, 35 per cent; corn starch, 26 per cent; lard 15 per cent; cod liver oil, 2 per cent; yeast (Anheuser-Busch Primary Dried Yeast No. 300), 1 per cent; bone ash, 1 per cent; and supplementary salt mixture (magnesium citrate, 55 per cent; ferric citrate, 27 per cent; manganese sulfate, 6 per cent, and copper sulfate, 12 per cent, by weight), 0.35 per cent, by weight. In addition, the animals received daily 0.5 gram NaCl and 0.1 gram KCl per kilogram body weight. Vitamin supplements were added to the yeast in the diet so that when fed at a level of 80 calories per kilogram the daily vitamin intake per kilogram body weight was: thiamine, 0.15 mgm.; riboflavin, 0.031 mgm.; nicotinic acid, 0.25 mgm.; calcium pantothenate, 0.20 mgm.; and pyridoxine, 0.015 mgm.

³ A uniform dose of 25 mgm. per kgm. of nembutal (pentobarbital sodium, Abbott) was used for anesthesia.

⁴ The low protein diet was identical with the basal diet except that the casein was replaced by an equivalent amount of glucose.

The total circulating plasma protein and total circulating plasma albumin were calculated by multiplying the plasma volume in cubic centimeters by the grams of protein per cubic centimeter. Similarly, the total circulating hemoglobin was calculated from the determined hemoglobin concentration and the calculated blood volume. The amounts of total plasma protein and plasma albumin removed from the circulation by the bleeding and by the withdrawal of blood for the chemical determinations were estimated by multiplying the cc. of plasma withdrawn (cc. whole blood withdrawn $\times \frac{100 - \text{hematocrit}}{100}$) by the grams of plasma protein or plasma albumin per cc. In a similar manner

the amount of hemoglobin removed from the circulation was estimated by multiplying the cc. of whole blood withdrawn by the grams of hemoglobin per cc. of blood. The amounts of these constituents restored to the circulation during the given test period were estimated by deducting from the grams of total plasma protein, plasma albumin and hemoglobin removed, respectively, the differences of the calculated amounts (control period values minus experimental period values) of total circulating protein albumin, circulating albumin and circulating hemoglobin.

RESULTS

The average blood changes which were encountered in normal dogs in 24 hours and in 96 hours following a) a single nonfatal hemorrhage, and b) a single nonfatal hemorrhage with immediate injection into the peritoneal cavity of the withdrawn blood, have been presented in table 1. The average blood changes which were encountered in dogs made hypoproteinemic by diet and treated in the same manner as the normal dogs have been presented in table 2.

Changes of the plasma protein concentration. 1) HEMORRHAGE. From the data presented in tables 1 and 2 it will be seen that 24 hours following a loss of blood amounting to 25 per cent of the control blood volume (during which period neither food nor water was allowed), the plasma volume was found to approach the control level. When the animals were allowed food and water following the 24-hour period and observed again 96 hours after the hemorrhage, the values for the plasma volume were found to be greater than the corresponding control figures. It will be seen that the plasma volume changes which were encountered in the animals subjected to bleeding and injection into the peritoneal cavity of the withdrawn blood are quite similar to those observed in the animals subjected to hemorrhage alone.

That initially, fluid in excess of protein is returned to the circulation following hemorrhage is evidenced by a lowered plasma protein concentration. If the difference between the measured amounts of total circulating plasma protein (control value minus experimental value) is deducted from the amount of total plasma protein removed as the result of bleeding, the result gives a measure of the amount of total plasma protein which entered the plasma during the given test period. A calculation based on the average data obtained with normal dogs (table 1), revealed that of the 7.9 grams of total plasma protein removed by bleeding, 3.1 grams (or 39 per cent) had been returned to the circulation in 24 hours after the hemorrhage. Similarly, in 96 hours after the hemorrhage the normal dogs were found to have added an average of 7.5 grams of total plasma protein (about 85 per cent of the total plasma protein removed) to the plasma. By treating the data for plasma albumin in the same manner, it was found that 1.8 grams of albumin were restored in 24 hours, and 4.3 grams in 96 hours, representing, respectively, about 36 and 76 per cent of the albumin removed during the bleeding.

A similar analysis of the data obtained on animals made hypoproteinemic by

diet (table 2) revealed that in 24 hours following hemorrhage 3.1 grams of total plasma protein and 2.0 grams of albumin had been added to the plasma. These figures represented a restoration of about 57 and 74 per cent, respectively, of the total plasma protein and plasma albumin removed by the bleeding. Observations which were made 96 hours following the hemorrhage indicated a still further restoration of plasma protein. That is, 6.7 grams of total plasma protein (representing about 92 per cent of the total protein removed) and 3.8 grams of albumin (representing about 109 per cent of albumin removed) were found to have been added to the plasma.

TABLE 1. AVERAGE BLOOD CHANGES 24 AND 96 HOURS AFTER A SINGLE NONFATAL HEMORRHAGE IN NORMAL DOGS

	HEMORRHAGE ¹				HEMORRHAGE AND INJECTION			
	Control (4)	24 hours	Control (4)	96 hours	Control (6)	24 hours	Control (4)	96 hours
Body weight, kgm.	10.55	9.90	10.55	10.45	12.48	12.09	12.70	12.58
Plasma withdrawn, cc.	136		153		137		139	
Hematocrit, per cent	46.6	45.5	46.6	40.1	51.6	47.5	54.7	49.2
Plasma volume, cc.	538	474	538	584	549	543	560	576
Total plasma protein, grams/100 cc.	5.79	5.55	5.79	5.12	6.22	5.83	6.06	5.31
Plasma albumin, grams/100 cc.	3.69	3.52	3.69	3.18	3.39	3.26	3.49	2.93
Hemoglobin, grams/100 cc.	13.6	13.2	13.6	11.5	14.6	13.7	15.6	14.1
Albumin/globulin ratio	1.76	1.73	1.76	1.64	1.20	1.27	1.36	1.23
Total circulating plasma protein, grams	31.2	26.4	31.2	29.9	34.2	31.6	34.0	30.6
Circulating plasma albumin, grams	19.9	16.7	19.9	18.6	18.6	17.7	19.5	16.8
Circulating hemoglobin, grams	137	115	137	112	166	142	193	160
Total plasma protein removed, grams	7.9		8.8		8.5		9.4	
Plasma albumin removed, grams	5.0		5.6		4.6		5.4	
Blood hemoglobin removed, grams	35		39		41		51	
Total plasma protein restored, grams		3.1		7.5		5.9		6.0
Plasma albumin restored, grams		1.8		4.3		3.7		2.7
Blood hemoglobin restored, grams		13		14		17		18

¹ Twenty-five per cent of the measured blood volume withdrawn. No food or water was allowed during the first 24 hours post-hemorrhage. The numbers within the parentheses indicate the number of animals.

2. HEMORRHAGE AND INJECTION OF BLOOD. The data obtained on the dogs subjected to bleeding and the immediate injection into the peritoneal cavity of the withdrawn blood were treated in the same manner as indicated above. In normal dogs (table 1) there were found to be on the average 5.9 grams of total plasma protein and 3.7 grams of albumin added to the plasma in 24 hours. These figures represented a restoration of about 70 and 80 per cent, respectively, of the total plasma protein and plasma albumin removed from the circulation by the bleeding. In the dogs made hypoproteinemic by diet, it was found (table 2) that in the 24-hour period 4.8 grams of total plasma protein (representing about 80 per cent of the total protein removed) and 2.6 grams of albumin (representing about 93 per cent of the albumin removed) had been returned to the plasma. It will be noted, therefore, that when

the withdrawn blood was injected immediately into the peritoneal cavity, greater amounts of total plasma protein and plasma albumin were found to be returned to the circulation in 24 hours. In other words, it would appear that the plasma proteins absorbed from the peritoneal cavity had contributed a share to the initial restoration of the plasma proteins.

In the preceding section it was noted that during the interval 24 to 96 hours, the animals subjected to hemorrhage alone continued to add plasma proteins to the circulation. Consequently, at the time of the 96-hour observation the amounts of the total plasma protein and plasma albumin restored were found to have approached the respective amounts removed. In contrast, the normal animals (table 1) sub-

TABLE 2. AVERAGE BLOOD CHANGES 24 AND 96 HOURS AFTER A SINGLE NONFATAL HEMORRHAGE IN DOGS MADE HYPOPROTEINEMIC BY DIET

	HEMORRHAGE ¹				HEMORRHAGE AND INJECTION			
	Control (4)	24 Hours	Control (3)	96 Hours	Control (3)	24 Hours	Control (4)	96 Hours
Body weight, kgm.	9.41	8.90	10.84	10.84	11.09	10.80	10.12	10.13
Plasma withdrawn, cc.	114		149		136		139	
Hematocrit, per cent	47.6	41.0	50.1	41.0	44.0	41.0	43.0	39.4
Plasma volume, cc.	407	401	465	517	522	506	462	496
Total plasma protein, grams/100 cc.	4.72	4.22	5.00	4.39	4.43	4.34	4.28	4.03
Plasma albumin, grams/100 cc.	2.36	2.23	2.43	2.25	2.07	2.10	2.16	1.96
Hemoglobin, grams/100 cc.	13.5	12.2	14.0	11.7	12.1	11.6	12.0	11.0
Albumin/globulin ratio	1.00	1.12	0.95	1.05	0.88	0.94	1.02	0.95
Total circulating plasma protein, grams	19.2	16.9	23.2	22.6	23.1	21.9	19.8	20.0
Circulating hemoglobin, grams	9.6	8.9	11.3	11.6	10.8	10.6	10.0	9.7
Circulating plasma albumin, grams	105	83	130	103	113	99	97	90
Total plasma protein removed, grams	5.4		7.3		6.0		6.1	
Plasma albumin removed, grams	2.7		3.5		2.8		3.0	
Blood hemoglobin removed, grams	29		41		29		29	
Total plasma protein restored, grams		3.1		6.7		4.8		6.3
Plasma albumin restored, grams		2.0		3.8		2.6		2.7
Blood hemoglobin restored, grams		7		14		15		22

¹ See footnote to table 1.

jected to the bleeding and injection procedure failed to add more plasma protein to the circulation during this interval. Thus, the observations made 96 hours following the hemorrhage and injection revealed that 6.0 grams of total plasma protein and 2.7 grams of albumin had been added to the plasma. These figures represented a restoration of about 64 and 50 per cent, respectively, of the total plasma protein and plasma albumin removed by the bleeding, and were lower than the corresponding percentage figures observed for the 24-hour period. Observations made 96 hours following hemorrhage and injection in dogs made hypoproteinemic by diet (table 2) revealed some further addition of plasma protein. However, the percentage restorations of the total plasma protein and plasma albumin at this period were also approximately similar to those encountered when the hypoproteinemic animals were subjected to hemorrhage alone.

The results of the observations which were carried out 24 hours following the hemorrhage indicate that when the withdrawn blood is transferred directly to the peritoneal cavity, the absorbed plasma proteins do contribute to some extent to the initial restoration of the plasma protein to the circulation. It would appear, however, that the absorbed plasma proteins enter into an equilibrium with the supply of proteins normally called upon to restore the plasma protein deficit. Consequently, when the observations were made several days following the hemorrhage, the restoration of the plasma proteins was similar in the two groups of animals.

Changes of the hemoglobin concentration and the hematocrit. The data presented in tables 1 and 2 show that with the re-expansion of the plasma volume following the hemorrhage there was a decrease of the blood hemoglobin concentration and the hematocrit. If the percentage changes from the respective control levels are considered, it will be found that in the animals subjected to hemorrhage alone, the deficits of the average hemoglobin concentration and of the hematocrit were greater at the time of the 96-hour than at the 24-hour period of observation. On the other hand, the animals subjected to hemorrhage and injection of the withdrawn blood exhibited comparatively smaller percentage changes in the deficits of the hemoglobin concentration and the hematocrit during the same interval. This was particularly true in the animals made hypoproteinemic by diet.

These findings suggested that the circulating red cell volume was better sustained in those animals in which the blood was injected into the peritoneal cavity and indicated that with the absorption of the blood, red blood cells had been added to the circulation. In order to determine whether the absorption of blood from the peritoneal cavity contributed to the circulating hemoglobin, the amount of blood hemoglobin restored during the given test periods was calculated in the same manner as was the restoration of the plasma proteins. Such a calculation revealed that in normal dogs 13 grams of hemoglobin had apparently been returned to the circulation 24 hours following hemorrhage (table 1), and seven grams of hemoglobin in the case of the dogs made hypoproteinemic by diet (table 2). When the volume of circulating red blood cells was calculated on the basis of the hematocrit readings, the result indicated that the observed red blood cell volume was greater than the volume expected. That there was an apparent increase in the red blood cell volume during the 24-hour interval following a single non-fatal hemorrhage is a surprising finding, since in a previous study employing unanesthetized dogs (9) the changes of the hematocrit indicated an additional loss of red blood cells from the circulation, rather than a gain. In addition the work of Huber (10) had indicated that following hemorrhage in unanesthetized, normal, splenectomized and sympathectomized dogs, red cells disappear into certain regions of the vascular bed where they are withdrawn from the active circulation. In the present study the hemorrhage was induced in animals under sodium pentobarbital anesthesia and the difference in results may probably be attributed to the anesthesia. To test this point an unanesthetized normal dog was subjected to the standard hemorrhage of 25 per cent of the measured blood volume. Observations carried out in 24 and 96 hours indicated an apparently greater loss of hemoglobin and of red blood cell volume from the circulation than expected on the basis of the amounts of these components removed by the bleeding.

That the changes of the hemoglobin concentration and the hematocrit following hemorrhage may differ in normal anesthetized and unanesthetized animals has been observed by other workers. For example, Carr and Essex (19) reported that intact dogs subjected to acute hemorrhage under sodium pentobarbital anesthesia showed a marked increase of the concentration of hemoglobin after the hemorrhage and at the end of 24 hours still had 82 per cent of the preanesthetic concentration of hemoglobin. On the other hand, these authors found that after splenectomy the dogs showed a marked fall of the concentration of hemoglobin following hemorrhage and after 24 hours had only 66 per cent of the preanesthetic concentration of hemoglobin. Further, Elman and Riedel (20) recently reported that when hemorrhage was produced in dogs under local anesthesia, hemodilution proceeded promptly; however, when sodium pentobarbital was administered intravenously at a time when hemodilution was in initial progress, the anesthesia apparently reversed the process and in some cases led to actual hemoconcentration.

The use of sodium pentobarbital anesthesia in the present experiments presents a complication. However, since the animals in the two groups of experiments were treated in the same manner (the only difference being that in one group the withdrawn blood was injected immediately into the peritoneal cavity), it would appear that any difference in the amount of hemoglobin or of red blood cells returned to the circulation can probably be attributed to absorption from the peritoneal cavity. In the experiments with normal dogs (table 1), it will be observed that 13 grams of hemoglobin were returned to the circulation in 24 hours, and 14 grams in 96 hours following hemorrhage. These figures represented about 37 and 36 per cent, respectively, of the amount of hemoglobin removed from the circulation by the bleeding. On the other hand, when the withdrawn blood was injected immediately into the peritoneal cavity, there were found to be 17 grams of hemoglobin returned to the circulation in 24 hours (representing about 41 per cent of the hemoglobin removed), and 18 grams of hemoglobin in 96 hours (representing about 35 per cent of the hemoglobin removed). In the normal animals, therefore, the injection of the blood into the peritoneal cavity had little or no effect on the restoration of blood hemoglobin following hemorrhage.

A somewhat different conclusion was arrived at when the data obtained on the animals made hypoproteinemic by diet were examined (table 2). Thus, it will be noted that in 24 hours following hemorrhage, 7 grams of hemoglobin, and in 96 hours, 14 grams of hemoglobin had been returned to the circulation. These figures represented about 24 and 34 per cent, respectively, of the hemoglobin removed from the circulation by bleeding. In those animals in which the withdrawn blood was injected into the peritoneal cavity, there were found to be 15 grams of hemoglobin returned to the circulation in 24 hours and 22 grams in 96 hours. These figures represented, respectively, about 52 and 76 per cent of the hemoglobin removed by bleeding. Therefore, in the dogs made hypoproteinemic by diet, it would appear that the absorption of blood from the peritoneal cavity was associated with some restoration of the hemoglobin following hemorrhage.

In passing, it might be remarked that an analysis of the data relative to changes of the circulating red cell volume supported the findings based on hemoglobin.

That is, in comparison with the changes encountered when the animals were subjected to hemorrhage alone, in normal dogs there was no increase in circulating red cells associated with the absorption of blood from the peritoneal cavity. In dogs made hypoproteinemic by diet, however, there was a greater return of red cell volume, the percentage return being appreciably greater at the time of the 96-hour than at the 24-hour period of observation.

Urine nitrogen elimination. The urine total nitrogen elimination was examined in a number of the animals to determine whether the absorption of the withdrawn blood injected into the peritoneal cavity was accompanied by an increased nitrogen excretion. In table 3 are presented the average data for the urine total nitrogen, expressed in terms of grams of nitrogen per kilogram body weight per day, which were encountered for a three-day period immediately prior to hemorrhage, and for

TABLE 3. AVERAGE TOTAL NITROGEN EXCRETION IN THE URINE OF DOGS FOLLOWING HEMORRHAGE¹
(Values are expressed in terms of the control body weight)

COMMENT	BODY WEIGHT	BLOOD N WITHDRAWN	URINE TOTAL N PER DAY			
			Prehemor- rhage	Posthemor- rhage—days		
				0-3	3-6	
	kgm.	grams per kgm. body weight				
<i>Normal Diet</i>						
Hemorrhage (4)	10.6	0.69	0.34	0.27	0.32	
Hemorrhage and injection (3)	12.2	0.70	0.26	0.26	0.28	
<i>Low protein diet</i>						
Hemorrhage (3)	8.5	0.63	0.15	0.16	0.11	
Hemorrhage and injection (4)	10.1	0.55	0.09	0.11	0.10	

¹ See footnote to table 1.

two three-day posthemorrhage periods. The grams of nitrogen per kilogram body weight removed from the circulation by bleeding (and returned to the peritoneal cavity in one group of animals) are shown in column 3 of the table. It will be observed that there were no significant differences in the nitrogen excretion in the corresponding groups of injected and noninjected animals.

DISCUSSION

Admittedly, the present study revealed no dramatic differences in the changes of the plasma volume, plasma protein concentration and the hematocrit between the injected and noninjected animals following hemorrhage. This was a surprising finding in view of the evidence that an animal's own blood when placed in the peritoneal cavity is absorbed rather promptly and, further, that red cells labeled with radio-iron can be detected in the circulation shortly after being injected into the peritoneal cavity.

By comparison of the changes of the circulating plasma proteins obtained in

animals subjected to hemorrhage with those of animals subjected to hemorrhage and injection of the withdrawn blood, it would appear that the proteins of the absorbed plasma contributed in part to the initial return of plasma protein to the circulation.

Why the absorption of the cellular elements of the blood injected into the peritoneal cavity was associated with some increase of circulating hemoglobin and red blood cells in the case of animals made hypoproteinemic by diet and not in the normal animals is not clear. During the period of observation there was no evidence of jaundice indicating erythrocyte destruction. An analysis of the total nitrogen of the urine for periods as long as six days after the hemorrhage revealed no increased nitrogen elimination. In the normal dogs, therefore, it appeared as though the absorbed erythrocytes were withheld from the active circulation.

SUMMARY

Normal dogs and dogs made hypoproteinemic by diet were subjected under sodium pentobarbital anesthesia to a single nonfatal hemorrhage; in certain of the animals the withdrawn blood was transferred directly into the peritoneal cavity. Since the animal's own blood when injected into the peritoneal cavity is absorbed rather promptly, it was felt of interest to determine *a*) whether or not the proteins of the absorbed plasma contribute measurably to the initial restoration of the circulating plasma protein and *b*) whether or not the absorbed erythrocytes contribute measurably to the circulating red cell volume. By comparing the changes observed with those encountered when animals were subjected to hemorrhage alone, the following conclusions were drawn: 1) In normal dogs as well as in dogs made hypoproteinemic by diet, the proteins of the absorbed plasma contributed in part to the initial restoration of the circulating plasma protein. The plasma protein absorbed apparently contributed to the supply of proteins normally called upon to restore the plasma protein deficit. This was evidenced by the fact that observations made several days after hemorrhage revealed essentially the same restoration of plasma protein in the injected and noninjected animals. 2) During the period of observation, the absorption of erythrocytes in normal dogs was unaccompanied by a restoration of the circulating hemoglobin and the circulating red cell volume. In contrast, experiments with the dogs made hypoproteinemic by diet revealed that the absorption of erythrocytes was accompanied by some restoration of the circulating hemoglobin and the circulating red blood cells.

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SOME FACTORS IN THE REGULATION OF THE STROKE VOLUME¹

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THE classical experiments of Starling and his followers (1, 2), with a heart-lung preparation in which venous inflow was held constant, demonstrated that an increase in the aortic pressure reduced the stroke volume, and led to an accumulation of residual blood with dilation of the heart. This dilation, by increasing the presystolic fiber length, progressively increased the energy released by the ventricle, until the heart was again able to pump out, against the increased pressure, the original amount of blood per minute and per beat. Also, when the aortic pressure was reduced, the original Starling records show that the stroke volume suddenly increased and remained larger until the heart was reduced to the original size and the residual ventricular volume of blood was again small.

The workers with the heart-lung preparation also made it clear that epinephrine, and presumably other sympathetic influences, increased the oxygen consumption at a given diastolic size (3, 4). It has also been observed that these influences tend to increase the stroke volume in the open chest preparation (5, 6). There are therefore three well recognized mechanisms by which the stroke volume may be regulated: 1) the influence of nervous and hormonal stimulation; 2) the aortic pressure or resistance to cardiac ejection, which on increasing tends, other things being equal, to reduce the stroke volume and on decreasing tends to increase the stroke volume; and 3) the influence of increased diastolic size in increasing the energy released by the process of ventricular contraction. Wiggers and Katz (5) have shown that under ordinary circumstances the auricular pressure at the end of diastole is closely proportional to diastolic ventricular size, and that there are few influences which increase the resistance to relaxation on the part of the ventricle. Changes in filling pressure can therefore be related to similar changes in diastolic size, but only if an intact pericardium does not limit the extent of the dilation.

It may be assumed that these three factors which regulate the stroke volume, i.e., diastolic size, cardiac stimulation and aortic pressure, also play a rôle in regulating the stroke volume in the intact animal. The relative effectiveness of each would seemingly vary with different physiological conditions. There is obviously a range in which diastolic size and stroke volume are related (7, 8). The heart can pump out no more blood than returns to it during diastole. If the return is limited, diastolic size and stroke volume are necessarily limited in the same measure. If the return is large, stroke volume and diastolic size may also be large. As will be seen below, however, the stroke volume may also be small when the animal is at rest, and

Received for publication April 12, 1948.

¹ This work was supported by a grant from the Life Insurance Medical Research Fund.

the heart unstimulated, even though the diastolic size is such as to render the pericardium tense.

It is difficult to find in the literature a description of the regulation of the stroke volume which takes into account all three of the above factors as they operate in the intact animal. Emphasis upon the effect of diastolic size (filling pressure) has been made (7, 8). That the rôle of this factor is limited in acute response, perhaps by the non-distensibility of the pericardium, seems indicated by several investigations. Thus studies of cardiac size by x-ray show that the normal human heart is larger during recumbency than it is during exercise in the upright position, though the stroke volume is greater in the latter condition (9, 10). It is also said (11, 15) and we have made confirmatory observations that, when the animal is recumbent, the heart is slow and the venous pressure is in the high normal range, the pericardium limits the resting diastolic size of the heart.

The rôle of cardiac stimulation has been investigated by McMichael and Sharpey-Shafer (16) who have shown that the cardiac output in man is increased by an infusion of a small amount of epinephrine, even when mean blood pressure, atrial pressure and heart rate remain unchanged.

The rôle of reduced aortic resistance has been suggested by the observation (10) that the production of reactive hyperemia in the legs of man, like the opening of an A.V. fistula, produces an immediate increase in the stroke volume before any change in reflex cardiac stimulation or in right atrial pressure has time to occur.

Calculation of the stroke volume from the pressure pulse contour (18) in the dog allows the following of changes in this quantity from beat to beat, and the description of the immediate relationship of each beat to the venous pressure, and the aortic resistance, in the presence or absence of cardiac stimulation. Thus the separate and combined rôles of these three factors in regulating the stroke volume can be evaluated. We have employed simple techniques for changing the arterial and venous pressure level, viz, occlusion of the aorta, and release of that occlusion, stimulation of the cardiac and vagal nerves and injection of sympathomimetic drugs and of acetylcholine. Since all stroke volumes so derived are expressed as cc. per sq. M. body surface, all values calculated from these stroke indexes, such as flow per second and cardiac work, are similarly adjusted for body size.

During occlusion of the abdominal aorta, the calculation of the stroke volume was modified by omitting from the sum of uptakes that of the abdominal aorta, viscera and legs. Systolic drainage was calculated in the usual way whether the occlusion was effective or not. In some experiments a shunt was opened between the aorta and the abdominal vena cava. When this was done, the uptake of the leg arteries was omitted from the calculation, and in calculating drainage P was used instead of $P-20$. This is because with large tubes, such as the shunt, flow does not cease until the pressure approaches the venous level.

The work of the left ventricle was calculated as PQ , where P is the mean pressure during systole and Q the stroke volume. The kinetic work was regarded as being insignificant: also, a much larger source of error—that of using mean rather than integrated PQ products in evaluating the work, was neglected (19).

The peripheral vasomotor resistance R_v was calculated as $R_v = P-20/F$, where R_v is the vasomotor resistance in arbitrary units, P , the arterial pressure in mm. Hg, and F , the blood flow in cc./sq.M./sec. The term vasomotor resistance is used to distinguish it from the simple pressure-flow ratio which is usually termed total peripheral resistance. The reason for making this adjustment in the formula is that when pressures approach 20 mm. Hg, as during vagus stimulation, the flow lessens in

much greater proportion than does the pressure (20). Thus there appears a large increase in calculated total peripheral resistance which is due to the peculiarities of the flow of blood through capillary vessels rather than to a vasomotor change. When the revised formula is used, the figure for resistance remains about the same when the arterial pressure falls due to vagus stimulation. With an A-V shunt in the system, the usual relation P/F was used in calculating the resistance.

Dogs were employed in all experiments, with either morphine-sodium pentobarbital, morphine-urethane, or morphine-ether anesthesia. Arterial pressures were recorded optically from the ascending aorta by means of a sound passed down the left carotid artery. Similarly, venous pressures were recorded from the vena cava by a sound passed down the jugular vein or from the left ventricle through a sound passed by the aortic valves from the aorta.

RESULTS AND DISCUSSION

Effects of increased aortic pressure. The pressure against which the heart worked was suddenly increased by occlusion of the abdominal aorta at the diaphragm, for periods of 3-5 minutes. The immediate effect was usually a decided reduction in stroke volume and ventricular work (fig. 1 A, B). As a consequence of the reduced stroke volume, residual blood may be supposed to have accumulated in the heart to cause the recorded increase in left heart filling pressure (fig. 1 B). This, and the consequent increase in diastolic size, often (fig. 1 A) but not always (fig. 1 B), produced a small immediate increase in stroke volume and work. The almost negligible increase in these cardiac functions indicates that the ventricular dilation could not have been in any real sense compensatory. Both work and output usually remained at very low levels during the aortic occlusion in spite of the increased filling pressure.

During the course of the second minute of occlusion there was often a gradual increase in the work which the heart was able to do and a reduction of the peripheral resistance (fig. 1 B). The increased cardiac work cannot be laid to an increase in diastolic size because the filling pressure remained constant. The peripheral resistance change may have been a reflex adjustment to high pressure in the aortic arch and carotid sinus, and the small gradual increase in cardiac output an indirect reflection of this, or of a possible augmentation of coronary flow from high pressure.

The reflex vasodilation during the occlusion differs according to the anesthesia used. Hence a dog under morphine-sodium pentobarbital anesthesia can reduce the peripheral resistance but little, whereas the animal under morphine-ether can decidedly lower the peripheral resistance. Curiously enough, this same contrast is apparent in the basal resistance levels under the two anesthetic agents. Under sodium pentobarbital, the blood pressure tends to be high, the pulse relatively rapid and the peripheral resistance from 2 to 5 units. Under ether, pressures are moderately low, heart rate fairly rapid and the resistance from 0.6 to 2 units.

Response to reduced arterial pressure. When an aortic occlusion lasting 3-5 minutes was released, the arterial pressure fell precipitously as the blood flowed from the upper aorta to the nearly empty arterial bed below the point of occlusion (fig. 2). After this sudden fall had occurred, the rate of pressure fall during diastole still

remained greater than normal because during the occlusion there had been reactive vasodilation of that part of the arteriolar bed in which there had been no blood flow (fig. 2).

The first heart beat after release of the occlusion produced a very large stroke volume. The pressure pulse was smaller than before release of occlusion because of a) increased systolic drainage; b) participation of a larger arterial bed in the uptake;

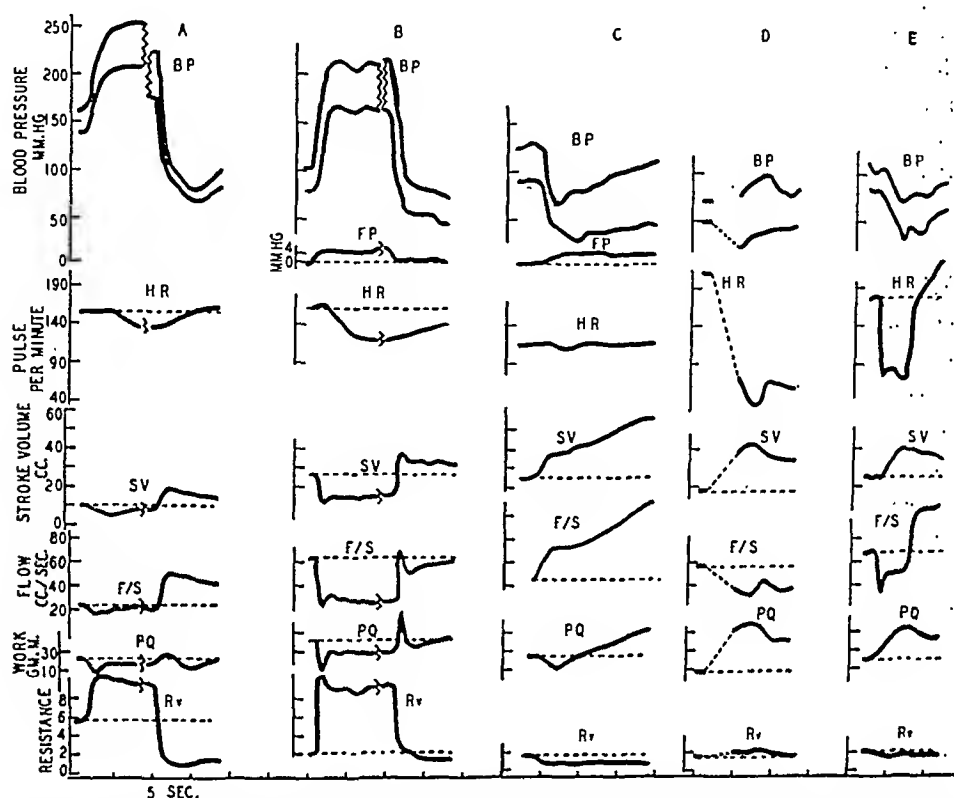


Fig. 1. BP = aortic blood pressure, systolic above, diastolic below; HR = pulse rate; SV = stroke volume, cc/M² body surface; F/S = blood flow per second, cc/M² body surface; PQ = left ventricular work per beat; Rv = vasomotor resistance = $\frac{(Pm - 20)}{F/\text{sec.}}$; FP = filling pressure of the left ventricle.

A, B—Occlusion of the abdominal aorta, and later release; morphine-sodium pentobarbital anesthesia. C—Opening of an abdominal aorta-vena cava shunt; morphine-urethane anesthesia. D—Stimulation of the peripheral end of the vagus; morphine-sodium pentobarbital anesthesia. E—Response to an injection of 10 $\mu\text{g/kgm. B.W.}$ of acetylcholine chloride; morphine-sodium pentobarbital anesthesia.

and c) the greater distensibility of the arteries at the lower than at the higher pressure ranges. Not only was the stroke volume abnormally large, but also there was a marked increase in the amount of external work, since the reduction in pressure was proportionately less than the increase in output.

This increase in stroke volume and external work was not considered to be related to any change in venous pressure or reflex change in the nervous or hormonal balance of the heart, for the change was immediate. Less than 150 msec. elapsed between the release of occlusion and the next beat, which showed the maximally

increased stroke volume. The response to suddenly lowered arterial pressure is best regarded as the simple mechanical result of a change in the relation between the tension developed by the ventricular myocardium and the pressure against which it works. As pressure decreased, a larger quantity of blood could be ejected by an unchanged contractile process.

Succeeding beats after the first, following release of occlusion, took a variable course (fig. 1 A B). In most cases the stroke volume diminished as blood was shifted from the venous reservoir to the dilated periphery. There seems little doubt that the venous return was the limiting factor in regulating the cardiac output at this time. Nevertheless the stroke volume was usually, and the external work often, increased above normal, while the venous pressure was reduced by the acceptance of blood in the dilated periphery. In all cases the stroke volume and often the external work were greater after than during occlusion.

The sudden increase in stroke volume upon the release of aortic occlusion requires that there be a considerable amount of residual blood in the ventricle at the time the change takes place. As a result of the high aortic pressure, during the occlusion period the left ventricle put out less blood than normally. The accumulation of residual blood increased the filling pressure. In the experiment cited, enough blood was so stored that the stroke volume was increased three-fold when the resistance was decreased.

Reducing the arterial pressure from the normal range down to very low levels gave changes of the same sort as those described above (fig. 1 C). This experiment was performed by having a large shunt between the abdominal aorta and the vena cava. On opening this shunt, the peripheral resistance fell rapidly and the stroke volume increased before any adjustment of filling pressure or reflex stimulation could have occurred.

There are two important differences. One is that on release of the occlusion, the stroke volume increased from 16 to 51 cc. On opening the shunt, the increase was only from 24 to 36 cc. This presumably means that less residual blood was stored in the ventricle in the latter case, and hence there was a smaller increase in the stroke volume with a similar drop in pressure.

After opening the shunt, the stroke volume gradually increased. This is in contrast to the decrease in stroke volume which occurred a few beats after the opening of an aortic occlusion. The increase in stroke volume following the opening of the shunt was due to an increased diastolic filling. The blood passing through the shunt returned at once through the vena cava, whereas after aortic occlusion it remained in the dilated peripheral bed. The filling pressure of the left and right ventricles rose after opening the shunt, and fell after release of the occlusion.

In these and related experiments the ventricle, working under normal conditions, retained a volume of blood from a half to a full stroke volume in size. When working against a high arterial pressure, this volume of residual blood is increased; when the pressure is reduced, this volume is lessened. The experiments illustrated were performed under sodium pentobarbital anesthesia, and from direct observations made on similar animals we may conclude that the ventricle in the pre-occlusion period was not filled to the limit of the pericardium. Only when the arterial pressure was

elevated did the ventricle dilate, and then not always to the pericardial limit. Under ether anesthesia, the changes following release of the occlusion, while showing similar trends, were not so dramatic. The heart size was large at the beginning of the experiment and did not increase greatly as a result of the occlusion. Under ether the reflex vasodilation, which occurred only in small measure under pentobarbital, was very pronounced. This caused the peripheral resistance to fall nearly to the pre-occlusive level, and the stroke volume to increase to a high level. The stroke volume reached upon release of occlusion was as great as that illustrated in figures 1 A and B but the net increase over that during occlusion was much smaller.

Effect of stimulating the vagus and cardiac accelerator nerves. Stimulating the accelerator nerves, in the few experiments we have performed, caused an increase in the output and work per second. These figures calculated per beat remained unchanged.

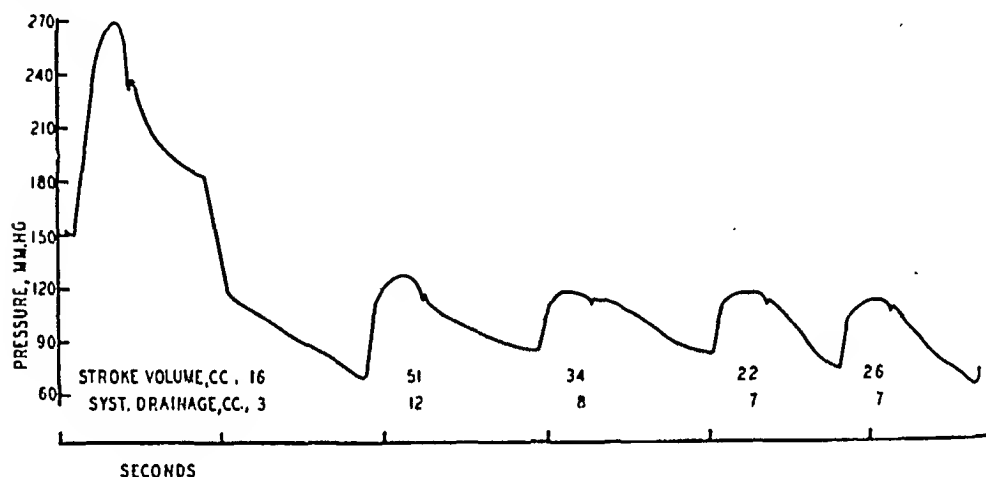


Fig. 2. AORTIC PRESSURE PULSE CONTOUR CHANGES following release of an occlusion of the abdominal aorta.

Slowing the heart by peripheral vagus stimulation caused a marked increase in the stroke volume and work per beat. The flow and work per second were greatly reduced (fig. 1 D).

Acetylcholine, (fig. 1 E) had an action similar to vagus stimulation during the early inhibitory stage. The reduced peripheral resistance and reflex cardiac stimulation resulted in a decided increase in output and work per minute. The stroke volume and work per beat were often increased or remained normal (21).

Effect of certain sympathomimetic drugs. The sympathomimetic drugs which we have used all increase the action of the heart. Some, in addition, caused peripheral constriction (e.g., epinephrine). Others had little or no effect upon the periphery and exerted their pressor effect through cardiac stimulation (e.g., ephedrine and first administration of Priscol), while others produced a peripheral dilation and moderate or severe pressure fall, depending upon the action of the drug upon the heart (isopropyl arterenol, second administration of Priscol).

The results of intravenous injections of ephedrine (fig. 3 A) and Priscol² (fig. 3 B)

² We are indebted to Dr. F. F. Yonkman for the Priscol and Dr. M. Sahyun for the n-iso-propyl-arterenol.

illustrate the effect of drugs in which the cardiac action is predominant. The vaso-motor resistance remained unchanged with Priscol, and the small increase in resistance which was seen after ephedrine came late and was not responsible for the primary rise in blood pressure. The initial response to both of these drugs was an abrupt rise in cardiac output and a consequent rise in arterial pressure. The arterial pressure rose to figures which, on simple occlusion of the aorta, tend to restrict the cardiac output and the work of the heart. Under the influence of ephedrine and

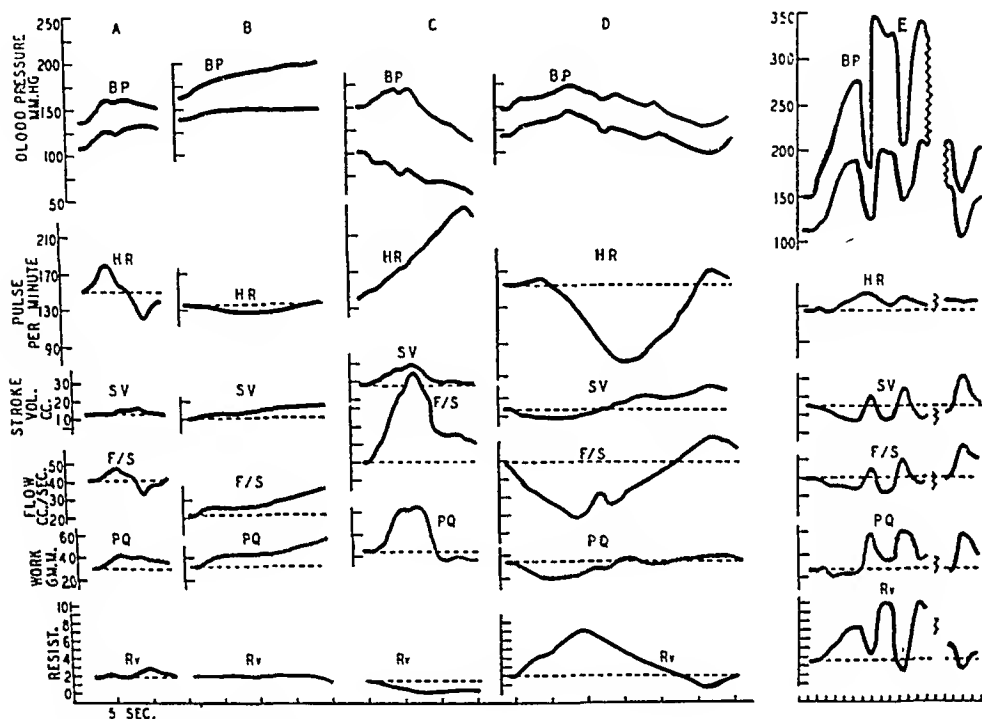


Fig. 3. Symbols as for figure 1.

A—Response to injection of 5 mgm/kgm. B.W. of ephedrine sulfate; morphine-sodium pentobarbital anesthesia. B—Response to injection of 10 mgm/kgm. B.W. of Priscol (22); morphine-sodium pentobarbital anesthesia. C—Response to an injection of 2.5 µg/kgm. B.W. of n-isopropyl-arterenol (23); morphine-sodium pentobarbital anesthesia. D—Response to an injection of 5 µg/kgm. B.W. of epinephrine hydrochloride; morphine-sodium pentobarbital anesthesia. E—Response to an injection of 20 µg/kgm. B.W. of epinephrine hydrochloride; morphine-sodium pentobarbital anesthesia. The sudden pressure drops followed the opening of an abdominal aorta-vena cava shunt.

Priscol these quantities increased rather than decreased, indicating that the working ability of the myocardium was greatly enhanced by the drugs.

Isopropyl arterenol² also caused a marked increase of the stroke volume and of the work per beat (fig. 3 C). The heart accelerated, so that the flow and work per second were increased even more. After a few seconds, the drug acted upon the periphery to cause a dilation. As the peripheral resistance declined, the pressure and work were reduced. Blood evidently accumulated in the periphery to the detriment of venous return, and the stroke volume fell back to a figure similar to that which obtained before the drug was given.

Epinephrine has two antagonistic effects and the final results are variable. The drug stimulates the heart and increases the peripheral resistance. The tendency of the first is to increase the cardiac output and work; that of the second to decrease them. The net action of the drug will depend, then, upon whether it is more active on the heart or on the periphery. This in turn depends upon the species in which the drug acts. In man, for instance, the work of Allen *et al.* (24) indicates that epinephrine produces a generalized vasodilation in the muscles. There seems to be a reduction in total peripheral resistance of man (9). Experience with the flow meter in the hands of Dr. R. P. Ahlquist indicates that the muscle vessels dilate more, after epinephrine, in some species (cat) than in others (dog and rabbit) (25). Epinephrine then may produce a net rise, a net fall or no change in the peripheral resistance of the body as a whole, depending on species differences in the peripheral action of the drug on the different arterioles of the body. If the peripheral effect is such as to have constriction and dilation balance out with no change in the peripheral resistance, the rise in blood pressure would be the result of cardiac stimulation (cf. ephedrine and Priscol on the dog). The rise in blood pressure might be the result of a moderate net vasoconstriction and cardiac stimulation, or it might be due to an intense generalized vasoconstriction which actually restricts cardiac output and work. In other words, epinephrine may well have, in different species, as varying effects upon hemodynamics as do the several sympathomimetic amines in the dog.

In the dog, typical results from the literature, based on work on the open chest preparation using the cardiometer, show that the stroke volume increases (3, 6). According to measurements made in the intact dog, however, the initial effect is to diminish the cardiac output and the stroke volume (for literature, see 26). As seen in figure 3 D our results indicate a reduction in both the output and work of the heart as a result of a dose of epinephrine of $5 \mu\text{g}/\text{kgm}$. No dose from 1 to $25 \mu\text{g}/\text{kgm}$ caused a definite initial increase in either factor, though the effect often reversed itself in late stages of the drug response. Thus in figure 3 D there was at first a diminution in stroke volume and work, followed as the blood pressure fell by a decided increase in both of these quantities. With extreme vagal slowing, the stroke volume and work per beat might be increased earlier, but the flow and work per second remained low. It is true that the balance between the effect of epinephrine on the heart and on the peripheral resistance may be changed, in deteriorated dogs with low pressure levels, so that the drug may actually increase the stroke volume above the initial level. If the peripheral action of the drug should be prevented, then the cardiac action of epinephrine might be expected to become clearly manifest. The response to the drug would then be closely similar to that of isopropyl-arterenol (fig. 3 C).

On the basis of the reduced cardiac output, it might seem that in the dog epinephrine is useless as an adjuvant to the emergency function of the sympathetic system. Stalling the effective action of the heart because of high blood pressure can hardly be a useful emergency response. The natural sequence of adrenal secretion, however, is muscular activity, whether in flight or in combat. This, through the action of local nervous mechanisms and the accumulation of metabolites causes vasodilation in the muscle arterioles and acts hemodynamically like the opening of a shunt. The action of epinephrine on the heart causes it to beat more vigorously and,

with the lower resistance from opening up of the muscle blood vessels, either directly in some species or as a result of exercise in others, to put out blood more effectively.

A counterpart of this normal phenomenon is seen in the experiment of figure 3 E. The sudden drops in pressure were due to the opening of a shunt which had been established between the abdominal aorta and the vena cava. Each time the shunt was opened, the stroke volume and cardiac work increased to figures well above normal.

Influence of the pericardium on stroke volume responses. It has been pointed out that whenever the arterial pressure is raised, the stroke volume of the heart is immediately curtailed. When, in a constant inflow heart-lung preparation, the aortic resistance is increased and the stroke discharge similarly reduced, the heart either dilates and compensates, once again delivering a normal stroke volume, or it overdilates and fails abruptly (1, 2). The heart in the intact animals did neither but continued to pump steadily a relatively small stroke volume against a high arterial pressure. Two explanations could be offered. The first would be that the pericardium prevented any large degree of compensatory dilation, and certainly prevented extreme dilation and decompensation. The second would be that venous inflow, in these experiments, was not sufficient to allow full dilation. A preliminary series of experiments was made in which the chest was opened under artificial respiration. The ribs were separated with the animal on its side, so that there would be a clear view of the heart and as little handicap to cardiac filling as possible. Occlusion of the aorta was performed and epinephrine injected, before and after removal of the pericardium.

The responses to occlusion fall into two patterns. When the animal showed a small heart, rapid pulse, small stroke volume, high peripheral resistance and low venous pressure before occlusion, the presence or absence of the pericardium did not appreciably change the response. The basic reason seems to be that for a rapid heart and a low venous pressure, dilation of the heart was not sufficient to produce compensation. On observation, the heart did not fill the pericardium when it was intact, nor did it exceed the volume of the pericardium when it was slit open, even during the occlusion maneuver.

When, however, the stroke volume and flow were large, as under ether anesthesia, a demonstrable difference in response to aortic occlusion was seen. In the control period, the pericardial membrane was tense in systole and in diastole, and the pumping action of the heart consisted of an apexward movement of a A.V. groove in systole, with little medial movement of the lateral ventricular walls. This type of cardiac movement has been recognized as normal in the x-ray shadows of the resting unanesthetized dog (27).

On occluding the aorta, there was a small rise in venous pressure, but since the heart was already filling the pericardium there was very little or no increase in diastolic size. The stroke volume was greatly restricted and remained so until the peripheral resistance was lowered. After removal of the pericardium, the heart, on occluding the aorta, dilated well beyond the limits of the pericardium. The stroke volume depression was smaller than when the pericardium was intact and showed a prompt recovery to the initial level.

A similar contrast applied to the responses to identical doses of epinephrine with

pericardium intact and removed. The heart dilated to the pericardium in the one case, and well beyond it in the other. In the latter case, the stroke volume and work per beat were much greater than before pericardium removal.

The experiments indicate that the pericardium plays a rôle in the failure of the ventricle to accomplish a compensatory dilation when faced with an acute increase in arterial pressure. It should be emphasized that all of the experiments presented here deal with the immediate cardiac response to large pressure changes. The results are not, therefore, applicable to such chronic problems as hypertension, where the heart is dilated, the pericardium increased in size, and the stroke volume returned to normal levels.

SUMMARY

Three important mechanisms are recognized for regulation of the stroke volume: 1) change in diastolic size of the ventricles as evidenced by change in filling pressure; 2) change in the resistance to ejection (aortic pressure); and 3) change in the degree of cardiac stimulation.

Working with the intact dog, our experiments indicate that an acute increase in the arterial pressure against which the heart must eject blood tends to reduce the stroke volume and usually to reduce the external cardiac work. This maneuver caused the heart to dilate, but when the pericardium is intact the dilation is not sufficient to produce a significant increase in cardiac function. Under some circumstances removal of the pericardium will allow sufficient dilation to improve the pumping action of the heart, but under other circumstances this is not true.

Decreasing the arterial pressure tends to increase the stroke volume and external work. This increase may occur immediately, i.e., before reflex adjustments or changes in filling pressure have had time to take place. The fact that the response may occur immediately necessitates the assumption that there is residual blood in the ventricle at the end of systole. A part of this blood can be ejected by a more complete emptying of the ventricle when the pressure is suddenly reduced.

The responses to increased and decreased arterial pressure are regarded as simple mechanical results of varying the resistance to ejection in the face of a relatively unchanging contractile process.

Sympathetic and sympathomimetic stimulation of the heart increases its contractile activity. This may result in an increased stroke volume and an increase in external cardiac work, if the peripheral resistance is not so greatly elevated as to give rise to an overwhelming increase in arterial pressure. In this case output and work may be reduced.

In these experiments, changes in resistance to ejection (aortic pressure) and in myocardial stimulation are more important than changes in diastolic size (filling pressure) in the regulation of the stroke volume. The responses studied are to acute changes and hence are not relevant to the results of prolonged strain which give rise to cardiac enlargement and hypertrophy.

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VOLUME ELASTICITY CHARACTERISTICS OF THE HUMAN AORTA AND PREDICTION OF THE STROKE VOLUME FROM THE PRESSURE PULSE¹

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IT HAS been demonstrated that when the transmission time through the arterial tree, and the different volume-pressure and pressure-flow relations of the parts are taken into account, the stroke volume of the dog can be quantitatively derived from a central pressure pulse contour (1). Stroke volumes so calculated agree with those determined by the dye injection technique within an average of ± 8 per cent. This technique allows the following of stroke volume changes from pulse beat to pulse beat and hence is well adapted to problems where cardiovascular dynamics are abruptly altered.

An attempt to apply this approach to the human is beset with major difficulties. First, in a sample of street dogs of sound health, the age span is not large and cardiovascular pathology is infrequent. The volume-pressure relations of the aortas of a series of such animals, corrected for body size, are quite consistent. In the human, on the other hand, where ageing and atherosclerosis are a problem, the aortic volumes are quite variable.

Secondly, the contour of the pressure pulse in the dog can be taken from the ascending aorta itself. Needless to say, central pressure pulses are not directly recordable in the human, and whether the brachial pulse follows a central pulse closely enough so that it can be used to calculate stroke volumes from the pulse contour is unknown.

In the hope that a method of stroke volume calculation could be constructed for the human which, while lacking the quantitative consistency of the method for the dog, could give values approximating the actual, sufficiently to be of practical benefit, we have assembled stretch data on 44 human aortas, covering an age span from 8 to 89 years². For the most part these were accident cases.

METHODS AND RESULTS

At autopsy, the length of the aorta was measured *in situ* in three sections: a) from the aortic valves to the exit of the left subclavian artery, hereafter referred to as the 'arch'; b) from the subclavian to the diaphragm, the 'thoracic' aorta; and c) from the diaphragm to the aortic bifurcation into the common iliacs, the 'ab-

Received for publication February 11, 1948.

¹ This work was supported by a grant from the Life Insurance Medical Research Fund.

² We wish to express our gratitude to Dr. Casper G. Burn of the Department of Pathology, Long Island College of Medicine, for his assistance in the collection of this material.

dominal' aorta. The length of the arch was determined before the viscera were dislodged, and with the heart drawn down to the diaphragm. The length of the descending aorta was determined with aortic attachments undisturbed, but after the respiratory and digestive organs were removed. Next, a segment from each of these three aortic parts was marked off and measured. These segments were then removed and remeasured, to give the degree of retraction upon cutting free from body attachments. This retraction has varied from 2 to 15 per cent, being greater in the younger individuals.

The sections were kept in mammalian Ringer solution until they were subjected to stretch, which was always within 24 hours of the death of the individual. For the stretch measurements, a ring of from 5 to 10 mm. width was cut from each segment, its initial diameter measured and then it was subjected to 1000 grams tension, developed slowly, with the gain in half-circumference being recorded simultaneously. Two successive stretches on each ring were made, the values given on the second stretch being used for further calculation (2). The instrument used, the Scott serigraph, has been described earlier (2). The tension-length figures obtained were converted to pressure-volume equivalents, using the calculated *in situ* length of the segment.

The data obtained are given in table 1. All subjects known to have a history of hypertension, or with marked arteriosclerosis apparent at autopsy, are grouped separately. Aortic *in situ* lengths, when corrected for body size, showed good consistency without a progressive change with age. The only really aberrant case was number 25, a very obese individual. Hence there is little evidence of a compensatory gain in length accompanying either old age or hypertension.

The cross-sectional area of the ascending aorta and the volume capacity of the aorta showed the expected tendency for an increase with age. Individual variation, however, was large, with considerable overlap between age groups. Consistency within an age group was somewhat improved by correcting for body size, but even so it is quite impossible to predict from the age of an individual what the size of his aorta would be. As might be anticipated, the variability was greater in older than in younger individuals.

Variability in total aortic capacities was also large. As shown in figure 1, the means of no two age groups were significantly different from each other. This variability was greater in the older age groups, for here we find some aortas still 'young' in total capacity and others much enlarged. This same division can be noted in hypertensive individuals. In 5 of the 10 cases, the aortic dimensions were not increased. Three of these had rapidly fulminating hypertension with death in mid-age. Of the five with very large aortas, all had a long history of hypertension. Whether the enlargement seen in several of the older individuals in the nonhypertensive series might be attributable to hypertension cannot be answered from our data. Near terminal blood pressures might easily be a grossly misleading clue to the existence of hypertension.

It might be of interest to point out the discrepancies between the above data and those given by Hallock and Benson (3). These authors present a series of curves showing a progression in the characteristics of the aortic distensibility curve with age. Plotting percentage increase in volume at different pressure levels, they show that in

younger individuals the curve is somewhat sigmoid, with maximum distensibility around 80 mm. Hg. As the individual ages, the sigmoid character is lost, and maxi-

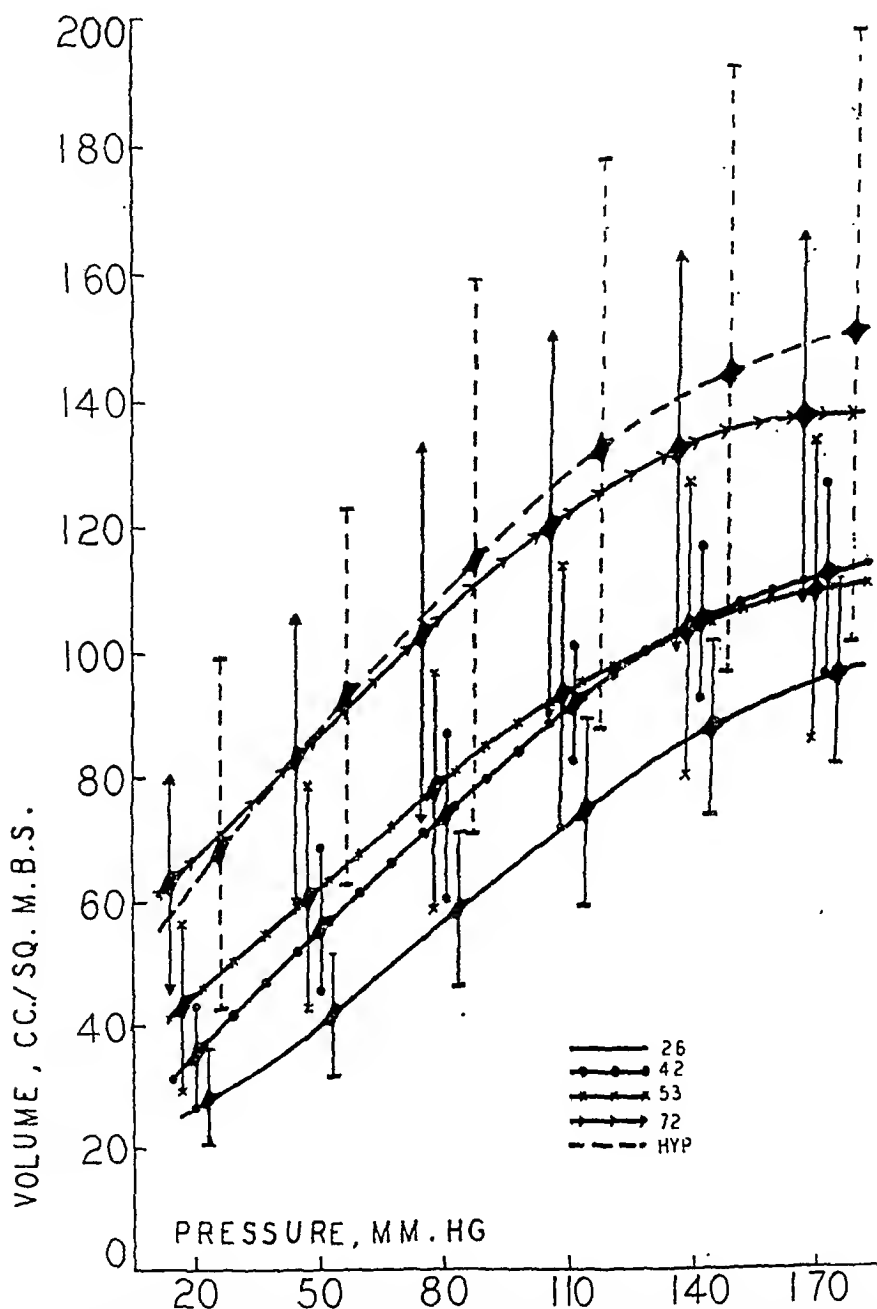


Fig. 1. INFLUENCE OF AGE ON THE VOLUME-PRESSURE RELATIONS OF HUMAN AORTAS. Each curve represents the average values for a given age group (table 1). Vertical lines represent standard deviations.

imum distensibility occurs at lower pressure levels. The number of cases represented by each of these curves, and the discrepancies within a series are not given. While a

similar trend is to be seen in the data of table 1, we need only point out that individual variation is such that 'young' aortas may be seen at 68, and 'old' aortas at 18 years of age!

For absolute volume changes, the curves of figure 1 are similar to those of Hallock and Benson for the middle age and older groups. Their curve for young individuals, however, showed a distensibility enough greater than with the older groups, so that while the zero pressure size was smaller, by the time 200 mm. Hg pressure had been reached, the young aortas were actually larger than the older. This we have not observed in any cases, even in the most distensible part of the aorta.

The data of Hallock and Benson were expressed as cc. volume per cm. length of thoracic aorta and bear no body-size correction. The data given above represent total aortic capacities corrected for body size. This difference alone cannot explain the disparity in results, however, for the same general relationship is also observed for the data of table 1, whether uncorrected for body size or expressed as cc. per cm. aortic length.

APPLICATIONS

In an attempt to predict the stroke volume of an individual from pressure pulses, two approaches might be used. We might, with Bazett *et al.* (4); take the pulse wave velocity as an index to the percentage increase in volume over a given pressure range. This percentage figure would have to be referred to an average aortic size, from which the absolute uptake would then be calculated. However, we would have to assume that pulse wave velocity can ever be an adequate index to the volume distensibility of the aorta, which in view of the hysteresis exhibited by aortic walls to rapid stretching, is hazardous (2)³. Even if a measured pulse wave velocity is taken to represent the proportional volume change in the aorta, the conversion from proportional to absolute volume change depends entirely upon the diastolic aortic capacity which is assumed. The data of table 1 show that these total capacities are quite variable, and the use of means may lead to considerable error. Further, different factors would have to be assigned to different age groups.

The correlation coefficient between the aortic capacity, corrected for body size, at, for example, 80 mm. Hg, and the volume gain from 80 to 110 mm. Hg is $-.05$. Hence there is no relation between diastolic capacity and volume gain. It can, therefore, be no more than coincidental if the volume uptake be predictable from pulse wave velocity. Parenthetically, it might be emphasized that volume distensibility defined as $\frac{\Delta V}{V \Delta P}$ has little usefulness other than the prediction of pulse wave velocity. This relation is meaningless when it is used, as has been done, to predict what pulse pressure would accompany a given stroke volume, or to predict the rate

³ In a previous publication (2), in which formulas were given for the relation between the volume uptake of the aorta and the pulse wave velocity, a factor F was assigned to cover the variable effect of hysteresis. As the formulas are stated, however, the factor should have been $1/F$. Hence formulas 3, page 545, should read: $V_p = \frac{0.357}{0.7} \sqrt{\Delta p \bar{V} / \Delta \bar{V}} = 0.51 \sqrt{\Delta p \bar{V} / \Delta \bar{V}}$ for the dog aorta and $V_p = 0.45 \sqrt{\Delta p \bar{V} / \Delta \bar{V}}$ for the human aorta.

TABLE I.—VOLUME-PRESSURE RELATIONS OF THE HUMAN AORTA

						AORTIC IN SITU LENGTH						ASC. AORTA CROSS-SECT. AREA SQ. CM./SQ. M.			AORTIC VOLUMES, CC./SQ. M.						VOLUME GAIN CC./SQ. M.					% CHG	
Case	Age	Sex	Pressure mm. Hg	Height, cm.	Weight, kgrm.	Arch	Thoracic	Abdominal	Total	Total/sq.M.	20 mm. Hg	80 mm. Hg	140 mm. Hg	20 mm. Hg	50 mm. Hg	80 mm. Hg	110 mm. Hg	140 mm. Hg	170 mm. Hg	20-50 mm. Hg	50-80 mm. Hg	80-110 mm. Hg	110-140 mm. Hg	140-170 mm. Hg	80-110 mm. Hg	Case	
1	8	F	?	122	23	4.5	9.5	13.5	27.5	31.5	1.5	3.0	5.2	12	20	30	42	58	70	8	10	12	16	12	40		
2	18	F	?	167	55	6	25	11	42	26	1.3	2.6	3.4	40	59	80	97	106	114	19	21	17	9	8	21	2	
3	22	F	?	165	55	8.5	17	14.5	40	26	0.7	1.5	3.3	19	32	44	61	82	93	13	12	17	21	11	39	3	
4	22	M	120/ 92	176	68	9.5	20	20	49.5	27	1.0	2.4	3.8	23	33	44	54	71	78	10	11	10	17	7	23	4	
5	24	M	?	168	64	9	21	12	42	24.5	0.9	1.9	2.6	25	40	55	70	75	78	15	15	15	5	3	28	5	
6	26	M	?	173	68	9	15	18	42	24	0.9	2.2	3.1	20	34	64	91	98	103	14	30	27	7	5	23	6	
7	27	M	125/ 80	173	68	8.5	17	14.5	40	22.5	1.9	3.5	5.4	33	49	67	87	103	115	16	18	20	16	12	29	7	
8	29	F	100/ 70	148	54	8	13	17	38	25.5	1.0	2.4	3.6	19	28	42	54	64	72	9	14	12	10	8	29	8	
9	30	F	150/ 60	168	63	6	22	18	46	27	1.1	2.4	3.7	23	35	49	75	84	90	12	14	26	9	6	32	9	
10	33	F	120/ 80	178	64	10	20	17	47	26.5	1.3	2.0	3.4	38	50	66	80	90	97	12	16	14	10	7	21	10	
Av.	26									25.5	1.1	2.3	3.6	27	40	57	74	86	93	13	17	17	12	7	29	Av.	
11	34	M	120/ 75	170	62	8	19	15	42	25	1.6	3.7	4.8	35	62	84	96	102	107	27	22	12	6	5	12	11	
12	38	M	110/ 70	180	77	10	20	16	46	23.5	1.3	3.0	4.2	37	47	57	82	102	109	10	10	25	20	7	44	12	
13	40	F	130/110	158	59	9	18	14	41	25.5	2.2	3.5	4.3	49	60	78	82	86	89	11	18	4	4	3	5	13	
14	41	M	?	183	63	10.5	22.5	15.5	48.5	26.5	1.4	2.3	2.5	41	70	84	102	124	141	29	14	18	22	17	23	14	
15	42	M	?	162	73	11	19	14	44	25	1.0	1.8	2.3	23	35	58	89	106	112	12	23	31	17	6	53	15	
16	44	M	140/ 80	183	102	10.5	22	16	48.5	22	2.0	2.9	3.5	44	62	81	94	107	112	18	19	13	13	5	15	16	
17	45	F	?	140	45	8	20	13	41	31	2.2	3.5	4.3	45	76	87	94	98	100	31	11	7	4	2	8	17	
18	45	M	130/ 85	173	45	9.5	20.5	13	43	28.5	1.2	3.0	3.2	34	47	67	86	94	98	13	20	19	8	4	28	18	
19	46	M	?	178	124	13	21	18	52	28	1.4	2.8	4.2	24	37	64	88	103	114	13	27	24	15	11	38	19	
20	46	M	?	168	68	9	18	23	50	22	1.5	2.6	3.6	40	70	97	114	127	135	30	27	17	13	8	17	20	
Av.	42									26.3	1.6	2.7	3.6	37	57	76	93	105	112	20	19	17	12	7	24	Av.	
21	49	F	?	173	82	7.5	22	18	47.5	24.5	1.1	3.0	4.0	25	36	50	64	72	76	11	14	14	8	4	28	21	
22	50	M	150/110	160	68	10	12	23	45	26.5	1.4	3.1	3.7	62	71	85	96	101	105	9	14	11	5	4	20	22	
23	50	M	122/ 80	178	73	12	18	15	45	23.5	1.4	3.1	3.8	48	76	93	111	125	132	28	17	18	14	7	5	23	
24	50	M	130/ 70	171	75	10	19	17	46	24.5	2.5	4.1	4.9	60	90	100	110	119	123	30	10	10	9	4	10	24	
25	52	F	180/ 82	155	52	11	16	16	43	28.5	1.1	4.7	5.0	30	44	58	71	79	83	14	14	13	8	4	23	25	
26	53	M	110/ 60	198	114	10	21	14	45	18	1.7	3.0	3.6	47	58	65	72	78	82	11	7	7	6	4	11	26	
27	55	F	130/100	170	82	9	20	14	43	22	0.9	2.0	3.0	38	45	65	85	92	98	15	7	20	20	7	6	27	
28	55	M	125/ 75	175	91	12	20	16	48	23	2.2	3.1	4.0	31	48	74	93	98	101	17	26	19	5	3	26	28	
29	58	M	150/ 70	163	91	10	23	14	47	24.5	1.0	3.4	3.5	63	84	103	123	141	145	21	19	20	18	4	20	29	
30	58	F	85/ 55	161	59	11.5	20	19	50.5	31.5	1.4	3.6	4.5	54	81	105	126	134	139	27	24	21	8	5	20	30	
Av.	53									24.6	1.5	3.3	4.0	46	63	80	95	105	110	17	17	15	10	4	17	Av.	
31	63	M	124/ 70	179	63	10	19	21	50	28	2.0	3.0	3.7	60	83	94	101	105	108	23	11	7	4	3	8	31	
32	65	M	150/ 74	165	83	12	15	16	43	22.5	2.0	4.3	4.6	57	65	74	83	91	96	18	9	9	8	5	12	32	
33	68	M	180/100	178	63	9	21	18	48	27.5	1.1	3.0	4.3	40	54	68	90	106	115	14	14	22	16	9	32	33	
34	68	M	160/ 90	166	65	11	19	17	47	27.5	2.8	4.9	5.5	70	93	103	112	120	125	23	10	9	8	5	9	34	
35	71	M	?	160	73	12	20	15	47	26	2.3	5.8	6.3	63	91	131	157	164	168	28	40	26	7	4	20	35	
36	76	M	160/ 70	168	62	8.5	15.5	25	49	29	0.9	1.3	1.9	89	115	148	158	165	169	26	33	10	7	4	7	36	
37	81	M	110/ 55	165	73	16	24	12	52	29	3.6	4.7	5.5	98	131	154	168	174	180	33	23	14	6	6	9	37	
38	89	F	200/100	153	52	11	18	14	43	29	3.9	5.6	5.9	81	96	110	123	132	136	15	14	15	9	4	12	38	
Av.	72									27.2	2.3	4.1	4.8	70	91	110	124	132	137	21	19	14	8	5	14	Av.	
Hypertensive Group																											
39	40	F	230/115	158	68	10.5	18	14	42.5	25	1.6	3.6	4.0	35	53	72	87	96	104	18	19	15	9	8	21	39	
40	45	M	280/170	172	73	12	20	17	49	26.5	1.7	2.3	2.7	34	40	46	52	59	65	6	6	6	7	6	13	40	
41	54	M	170/130	178	77	9	22	16	47	24	1.6	4.4	5.0	41	76	100	120	142	151	35	24	20	2	9	20	41	
42	55	F	220/180	168	77	14	18	12	44	23.5	3.1	7.7	8.7	96	126	178	191	209	214	30	52	13	18	5	7	42	
43	60	F	?	160	59	10	17	15	42	26.5	2.5	3.8	4.4	49	59	72	83	90	93	10	13	11	7	3	15	43	
44	60	M	200/130	180	66	14	23	17	54	29.5	2.6	7.1	7.7	87	132	177	190	201	209	45	13	11	8	7	44		
45	65	F	200/130	157	57	8.5	19	15	42.5	27.5	1.5	4.5	5.5	34	65	94	112	126	132	31	29	18	14	6	19	45	
46	66	M	?	165	49	9	17	17	43	28	1.9	5.6	6.7	69	84	111	135	151	160	15	27	24	16	9	22	46	
47	69	M	?	168	75	9.5	18.5	16.5	44.5	24	2.8	6.3	7.9	68	95	123	154	171	185	27	28	31	25	6	7	47	
48	88	M	?	168	50	15	17	16	46	29.5	3.4	5.7	6.9	117	140	161	173	181	184	23	21	12	8	3	25	48	
Av.	60									26.4	2.3	5.1	6.0	63	88	113	130	143	150	25	25	16	13	7	18	Av.	

of peripheral outflow or the rate of pressure fall which would take place in an arterial bed below a point of occlusion.

The second approach to predict stroke volumes would assume that the absolute volume uptake figures are constant enough, despite variations in aortic size, so that they could be used directly in evaluating total arterial uptake, and hence the stroke volume, from pressure changes. As shown by table 1, these absolute uptakes were more constant than were the percentage changes or total aortic capacities. For example, from 80 to 110 mm. Hg, the average volume uptake for all aortas save that of the 8-year old was 16 cc., with a standard deviation of 6.5 cc. or 41 per cent of the mean. The average percentage volume change was 20, with a standard deviation of

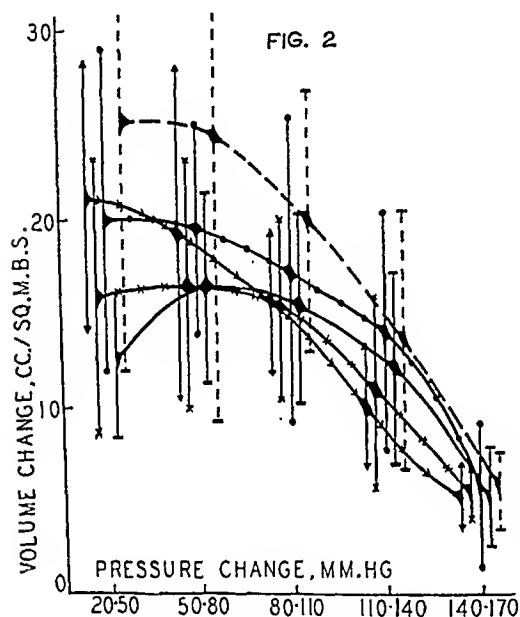


Fig. 2. VOLUME UPTAKE per 30 mm. Hg pressure increase of human aortas. Legend as for figure 1.

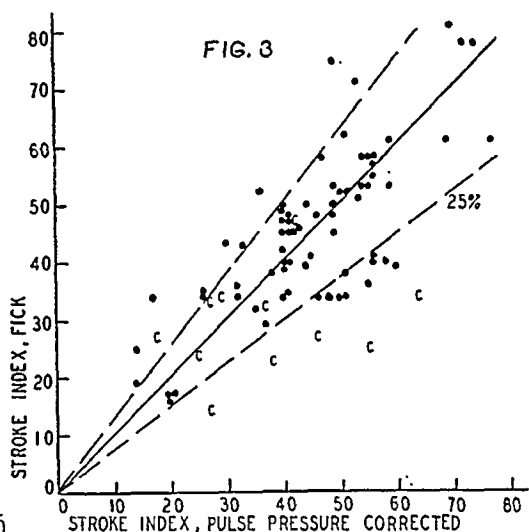


Fig. 3. COMPARISON BETWEEN THE FICK STROKE INDEX and the predicted index from pulse pressure corrected for changing arterial distensibility. C = congestive failure patient.

12 or 60 per cent of the mean. The same approximate ratio holds for other pressure intervals. Further, as shown in figure 2, there is little need to subdivide the series of aortas, for the standard deviation overlap rendered insignificant the observed differences in the means of respective age groups.

From the contour of the volume-pressure relations of the average aorta from table 1, we constructed a series of tables for volume capacity change of the arterial beds, much as such tables were constructed for the dog (1). The distensibilities of the various arterial beds were assumed to be qualitatively similar to those of the dog, and the actual volumes for these beds obtained by use of the ratio of arterial bed size to aortic volume size which was actually determined for the dog. The arterial volume of the legs was, however, made proportionately twice as large as that for the dog. A table of estimated transmission times was also drawn up. Using the same

method of calculation as used for the dog, we then analyzed a series of 83 brachial intra-arterial pressure pulse recordings, with their corresponding stroke volume values as determined by the direct Fick procedure, done a short time before the pressure recording. Sixty-two of these cases were supplied us by Dr. André Cournand from his series done at Bellevue Hospital, and the rest were from a series done at Georgia. In the entire series, 13 cases were from hypertensive individuals at rest and 70 were from normotensive individuals either at rest or during exercise. Thirteen patients were in congestive failure.

Both actual and predicted stroke volumes were expressed in terms of cc. per square meter body surface, i.e., as *stroke index*. The average discrepancy between stroke index derived from the contour method and from the Fick procedure was 20.9 per cent.

As a group, the congestive failure patients showed large pulse pressures as compared to the stroke indexes given by the Fick procedure. In five cases, the relationship was like the normals, but in the other eight the stroke indexes derived from the contour exceeded those given by the Fick procedure by as much as 100 per cent.

Why the cardiac patients show normal or greater than normal pulse pressures when the Fick stroke index is subnormal is a question which can only be speculated upon. Edema present in the cardiac patient would be reflected in a high value for estimated surface area and hence in a low-stroke index as determined by the Fick procedure. The difference between actual and predicted stroke index is too large to be explained on this basis, however. Three other explanations could be offered: *a*) that aortic regurgitation was present; *b*) that arterial distensibility is greatly reduced; and *c*) that for some unknown reason actual systolic drainage is much less than that calculated on the assumption that the drainage rate is proportional to existing pressure, and that systolic drainage bears the same relation to total drainage during a cycle as the time-pressure area under the systolic portion of a pulse contour bears to the area under the whole cycle.

As for the first, all cases in which diastolic murmurs were reported were omitted from this series. Is it possible that dilation of the ventricle would lead to valve incompetence, even though murmurs were not heard in the patients cited above? For the second, if we assume no regurgitation, then the aortas or arteries or both must

show abnormally low $\frac{\Delta V}{\Delta P}$ ratios. It is true that some cases in the autopsy series

given in table 1 show a distensibility as small as would have to be true for the cardiac patients. Why, however, should so many of the latter show values so far removed from the average? It is conceivable that a smooth muscle relaxation could accomplish such a distensibility change, but our experience with stretch measurements made on isolated aortic rings makes us doubt that a tone change could so radically alter the distensibility pattern at working pressure levels. Could the pressure of periarterial edema fluid render the arteries less distensible? The third possibility, that the drainage figure is in error, seems least likely. In some cases the arterial uptake, as deduced from the pulse pressure, is actually larger than the Fick stroke index. The addition of any sized systolic drainage volume will make the discrepancy worse.

If these eight cases of congestive failure are omitted from the series, the average deviation for the remaining 75 is 16.9 per cent. The correlation coefficient is 0.89. The failure to obtain better agreement than this can be attributed to at least two major factors. First, the brachial pressure pulse, modified as it is by its passage out the arterial bed, does not lend itself to strict quantitation. The dicrotic trough bears no necessary relation to the incisura of the central pulse, nor, judging from simultaneously recorded surface carotid and brachial pulses, is there any constant landmark on the predicrotic pressure fall which marks the remnant of the incisura. Since the whole contour calculation is based on a precise recognition of the end of systole and the pressure slope just preceding, the selection of an arbitrary landmark on the brachial pulses for the contour predictions leads to undefinable errors. Secondly, as has been shown, the human aorta is highly variable in its capacity change. There is enough variation in volume change between the aortas given in table 1 to more than account for the greatest discrepancy observed between predicted and actual stroke indexes. Unfortunately, the aberrant cases, with regard to aortic

TABLE 2. FACTORS FOR THE PREDICTION OF STROKE VOLUME, PER SQ. M. BODY SURFACE, FROM THE PULSE PRESSURE

PRESSURE	VOLUME FACTOR	PRESSURE	VOLUME FACTOR	PRESSURE	VOLUME FACTOR
<i>mm. Hg</i>	<i>cc.</i>	<i>mm. Hg</i>	<i>cc.</i>	<i>mm. Hg</i>	<i>cc.</i>
20	0	100	81	180	140
30	10	110	90	200	148
40	21	120	100	220	155
50	31	130	108	240	161
60	42	140	115	260	167
70	52	150	122	280	173
80	62	160	128	300	179
90	71	170	134		

distensibility in the autopsy cases, cannot be catalogued by the age or condition of the patient.

A recognition of the inapplicability of the brachial pulse to contour calculations, and the unavoidably large error inherent in the use of an average distensibility to represent all age groups and pathological states, led to the feeling that a prediction of stroke volume nearly as good as that obtained by the contour method could be obtained from the pulse pressure alone. It is obvious, however, that account must be taken of the changing distensibility of the arterial tree at different pressure levels. As shown in figure 2, the absolute uptake per unit pressure rise remains essentially constant from 20 to 110 mm. Hg, and then becomes less at higher pressure values.

Using the data of table 1, a series of factors were derived from aortic measurements and then increased proportionately until the average of predicted stroke indexes and of Fick indexes was the same. This procedure gives a series of factors from which the average stroke index can be predicted, and, at the same time, gives a smaller volume equivalent at higher pressure levels. The factors are given in table 2. Thus, if a brachial pulse tracing shows pressure values of 120/70 mm. Hg, the stroke index from table 2 would be $100 - 52 = 48$ cc. When these factors were

used for the entire series of 83 cases, the derived stroke index varied from the actual by an average of 25.2 per cent. If the eight cases of congestive failure be neglected, the discrepancy is reduced to 18.6 per cent, ranging from 0 to 56 per cent (figure 3). The standard deviation is 23 per cent; the correlation coefficient 0.79.

The fact that pulse pressure corrected in this manner gives a stroke index with an average error of but two per cent greater than that given by the calculation from the contour indicates that the more complex calculation involved in the latter procedure is not warranted. We are not, therefore, even publishing the tables for this calculation.

That the pulse pressure would show changes in the same direction as the stroke volume was first advanced by Erlanger and Hooker (5). While several workers (6-9) have employed $PP \times HR$ as a rough index to the cardiac output, no one would claim a quantitative relationship between the two. In fact, when tested on a heart-lung preparation, quantitation was not found possible (10), although the applicability of these findings to an intact animal is open to question (9).

Two reasons have been offered for a lack of correspondence between pulse pressure and stroke volume. The first lies in the changing arterial distensibility, which means that the volume equivalent of a given pressure will not be the same at high as at low pressure values. We have attempted to correct for this variation by the factors of table 2.

Several formulas have been advanced by which this correction for changing arterial distensibility is attempted by dividing a given pulse pressure by a constant or by an arbitrarily selected blood pressure value (11-14). All of these give a relation between pressure and volume of constant curvature unlike that shown in table 2. Hence each of them will give higher estimates of stroke volume at low pressure values than will the essentially straight line of our relationship. Whether the prediction at high pressure values is greater or less than that afforded by the factors of table 2 depends upon the actual curvature given by the individual formula.

Three of these proposed formulas have been applied to the series of 75 cases. In each case the constant employed was chosen to make the means of predicted and actual stroke indexes the same. For example, Bazett (13) used the quotient of $PP/(P_s)^2$, where P_s is the systolic pressure. This formula gave an average discrepancy of 34.2 per cent. If, following the suggestion of Bazett, the correction is applied only to pulses where the systolic pressure is greater than 100 mm. Hg, the average discrepancy was reduced to 28.6 per cent.

Furst and Soetbeer (12) used the expression PP/P_m , where P_m is the mean pressure level as derived by $Pd + \frac{PP}{3}$. The average discrepancy yielded by this relationship was 25.3 per cent. Liljestrand and Zander (14) also use PP/P_m , but P_m in this case is $\frac{Pd + P_s}{2}$. Their formula gave an average discrepancy of 24.1 per cent.

The assumption of a straight-line relation for all pressure values, while it over-

estimates high pressure pulses to a considerable extent, is almost as valuable as any of the above formulas. Hence the formula: Stroke index in cc. = $0.9 PP$, in mm. Hg, yielded an average discrepancy of 26.4 per cent.

The second reason for the lack of agreement between predicted stroke volumes and those obtained by the Fick procedure lies in the fact that the stroke volume is composed not only of the volume uptake of the arterial bed but also the volume lost by drainage during systole. Arterial bed volumes based upon aortic distensibilities will predict only the uptake. The factors of table 2 were set large enough to allow a prediction of actual stroke indexes and hence represent both uptake and a constant fraction for drainage. In the calculation from the pulse contours, the amount of systolic drainage was separately derived from the relationship of systolic to total pressure-time areas (2). In these pulses, the systolic drainage varied from 12 to 44 per cent of the stroke volume, averaging 25 per cent and with a standard deviation of 7 per cent. Our attempts to correct the volume factors of table 2 by the ratio of systolic to diastolic drainage areas, for the individual pulses, resulted in but a small improvement in prediction value. Hence the error involved in using an average relation of systolic drainage to total stroke volume is masked by the variation in distensibility between individual aortas.

SUMMARY

The capacities at different pressures of 48 human aortas were calculated from aortic lengths and the tension-length relations of rings cut from selected locations along the aorta. There was no statistically significant correlation between age and aortic length or absolute distensibility. Absolute aortic capacity increased with age and with the incidence of hypertension. Variability was so large, however, that neither age nor the existence of hypertension could be used to predict aortic size.

Since there is no correlation between aortic size and absolute distensibility, relative distensibility, i.e., $\frac{\Delta V}{V\Delta P}$, is more variable than is absolute distensibility, $\frac{\Delta V}{\Delta P}$.

A prediction of 75-stroke indexes from intra-arterial pulse pressures gave an average discrepancy of ± 26 per cent from the known Fick values. When the prediction was corrected for lessening arterial distensibility at high pressures, the discrepancy averaged ± 19 per cent. A complex calculation from the brachial pulse contour did not make the prediction appreciably better. These discrepancies are best explained in terms of varying arterial distensibility of different individuals.

Eight patients with congestive failure had small stroke volumes and aberrantly large pulse pressures. The explanation for this is not clear and they are not included in the above series.

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COMPARISON OF THE FICK AND DYE INJECTION METHODS OF MEASURING THE CARDIAC OUTPUT IN MAN¹

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IF WE can define a primary method of measuring the circulation rate or cardiac output as one in which empirical constants are not necessary in the calculation, then there are, if certain fundamental assumptions be granted, two such methods available. These are the direct Fick and the dye injection method.

In the Fick procedure, the blood flow is calculated from the formula

$$F = \frac{O}{(A - V)}, \quad \text{I.}$$

in which F is the blood flow in liters per minute, O is the oxygen consumption (or CO_2 production) in cc. per minute, and $(A - V)$ is the difference in oxygen (or CO_2) content between arterial and mixed venous blood in cc. per liter. The assumptions required are *a*) that no appreciable amount of the oxygen uptake disappears metabolically from the lesser circulation, and *b*) that a venous blood sample can be taken which is fully mixed. The former assumption is now universally accepted. The latter assumption is acceptable if the mixed venous blood sample is withdrawn through the catheter from the right ventricle or pulmonary artery; moreover, if proper precautions are taken, samples from the right atrium are also found to be well mixed (1-3).

With the injection method, the rate of blood flow is calculated from the relationship

$$f = \frac{I}{ct}; \quad \text{II.}$$

where f is the flow in liters per second, I is the amount of substance in mgm. injected into a vein, c is the average concentration (in mgm/l.) to which the injected substance is diluted by the blood stream during the first circulation, and t , the time in seconds required for this circulation of the dye.

To illustrate the meaning of this relationship we can imagine a stream of water flowing through a more or less complex model, but not recirculating (4). If dye is rapidly injected into the incoming stream, it will mix with the water and be carried through to the outlet. There it may be sampled by diverting *continuously* a small part of it through a needle. This flow is directed into tubes arranged to take samples every second. The rest of the stream containing dye is collected in a container. If the collections in the tubes and in the container are begun with the first appearance of the dye and stopped after all the dye has passed, and if the mean rate of flow is constant, then the average concentration of the samples in the tubes might be expected to be, and actually has been shown to

Received for publication January 29, 1948.

¹ This investigation was made possible by grants provided by the Life Insurance Medical Research Fund and the Commonwealth Fund.

be the same as the concentration of the dyed water in the container. Therefore, the average concentration of the samples in the tubes provides a measure of the rate of flow. For example, if 12 mgm. of dye be injected, and if it takes 30 seconds for the dye to pass, and if, during that time, 3 liters of dyed water are collected, then the 12 mgm. of dye will be found in the 3 liters of water, and the average concentration of dye in the sampling tubes (as well as in the container) will be 4 mgm.

per liter. If the rate of flow is calculated from the formula $f = \frac{I}{ct} = \frac{(12)}{(4 \times 30)} = 0.1$ liters per second or 6 liters per minute, the result is the same as the rate of flow measured directly from the dye concentration or from the volume of fluid in the container.

The application of the method was then tested in models so arranged that recirculation would occur before the dye on its first circulation had all passed the point of sampling (4). When samples are taken from such a model, the concentration curve rises, reaches a peak and then descends. If this curve is plotted on semilogarithmic paper, the descent is along a straight line, as it is in the straight flow models. Sometime later a break in this line, usually followed by a second rise in concentration, is clearly recognizable. This marks the beginning of recirculation, and extrapolation downward of the straight line portion of the descending limb, ignoring the secondary rise, makes possible the construction of a curve which would have obtained if the dye had not recirculated. When this curve is used to calculate the flow through models with recirculation, the result corresponds with the measured flow within the error of colorimetry.

When the dye is injected into the vein of an animal, the appearance of the concentration curve of arterial samples is very like that from the recirculating models. The straight line descent and the evidence of recirculation are clear. Moreover when in the body recirculation is not allowed, as when the heart and lungs are perfused with a stream of blood of the size of the cardiac output, the 'straight line' concentration curve obtains, as it does in the model experiments without recirculation (5), and the blood flow can be predicted from the concentration curve.

The further assumptions necessary to justify this method in calculating the cardiac output of man and animals are that the dyes brilliant vital red or T-1824 mix uniformly with the blood, do not leave the circulation and can be accurately determined. These assumptions are acceptable on the grounds that calculations from the heart-lung perfusion curve give calculated flows in agreement with those actually measured (5) and that measurement of the cardiac output in dogs by the dye curve agrees with that by the direct Fick method (6).

It is the purpose of this paper to compare in man, under a wide variety of conditions, measurements of cardiac output by the injection method with those by the Fick procedure. The work was carried out in two laboratories. Eighteen comparisons were made in the Georgia Laboratory and thirty in the Columbia Laboratory at Bellevue Hospital. The latter were the result of a cooperative venture in which two of the Georgia group (W.F.H. and J.W.R.) worked with the Columbia group in their laboratory.

Determination of cardiac output by the Fick method. The determination of cardiac output by the Fick method as used at Bellevue has been repeatedly described (1,2), but certain technical modifications have been introduced recently.

The catheter, #6 or #7F, was threaded into the heart through the basilic vein. In 24 of the 30 Bellevue cases samples of the mixed venous blood were taken from the pulmonary artery. In the other six mixed venous blood was taken from the outflow tract of the right ventricle. In the Georgia series, in 16 cases, the mixed venous blood was drawn from the ventricle, and in two from the right atrium. The arterial samples were drawn from an indwelling needle placed in the brachial artery of the other arm.

An anticoagulant and antiglycolytic solution was made up as follows: to 400

mgm. of sodium fluoride were added 3 ml. of heparin solution (Liquaemin, Roche-Organon, Inc.) and 3 ml. of distilled water. Syringes were prepared for blood sampling by greasing the plungers lightly. Several drops of the heparin-fluoride solution and a small drop of mercury were then introduced into the syringe, and with the syringe in the upright position the plunger was advanced completely. In this way all air was displaced from the syringe and the solution remaining in the tip served as an anticoagulant. Mixing was facilitated by the droplet of mercury.

Arterial and venous blood samples were started simultaneously with the beginning of the collection of the expired air. The arterial sample was taken slowly during the 1.5-minute period of expired air collection. To avoid clotting in the catheter, the duration of mixed venous blood sampling did not exceed one minute. After blood samples were drawn, the syringes were plugged with round toothpicks and were rotated slowly to prevent settling of the cells. Blood was transferred under pressure to one ml. Ostwald-Van Slyke pipettes through a 26-gauge needle inserted into the tip of the pipette. A small piece of rubber, pierced by the needle, made possible an air-tight seal.

Since available saponin used for hemolysis caused a heavy coagulum in the reaction chamber of the Van Slyke-Neill apparatus, a solution of Duponal C in similar concentration was substituted with good results.

The expired air was collected in a Tissot spirometer and a recording of the respiratory rate was made. The subject breathed air from a cylinder equipped with a demand valve.

The technique in the Georgia Laboratory differed in that the oxygen consumption was measured over 10 to 15 minutes by means of a Sanborn B.M.R. spirometer. Hence the patients breathed a rich oxygen mixture during both the Fick and dye determinations of cardiac output. The blood samples were drawn under freshly boiled oil and kept on ice. They were mixed manually and analyzed for oxygen with Van Slyke's Borax reagent (7).

Technique of the dye injection method. A measured amount of 0.5 per cent T-1824² was injected from a transparent 2 cc. insulin syringe, calibrated for the volume it delivered. Because the dense color of the dye solution obscured the end of the plunger, a line was etched on the plunger about one-fourth inch from the tip, permitting accurate filling of the syringe with dye when the etched mark on the plunger was aligned with one of the calibrations on the barrel. Repeated weighings of water ejected showed variations of the order of two per cent. When an indwelling needle was used in the vein, the capacity of the needle, as measured by water weight, was subtracted from the quantity delivered by the syringe. This was not necessary when the syringe was filled through the needle that was used for the injections.

The sampling device used in Georgia was similar to that already described (8). The receiving tubes were held snugly together on the circumference of a kymograph drum by a ring of rubber tubing. Another larger rubber tubing ring was placed around the kymograph just below, with a roll of gauze tucked between it and the tubes. Below the sampling tubes and their supports was a smoked surface on which

² 0.5 per cent T-1824 was obtained in 5 ml. ampoules from William R. Warner & Company New York, N. Y.

were signalled the instant of dye injection, and time in relation to the center of each sampling tube.

The kymograph to be used must have one feature, i.e., speed which is adjustable during the experiment. The most satisfactory device which we have used is the Stoelting kymograph³. This is a spring-driven machine with a magnetic brake, which permits speed to be increased or decreased without stopping the drum.

It often happens that flow through the sampling needle slows down so that an insufficient amount of blood flows into the tubes when the samples are taken at a rate of one per second. It is then necessary to change the kymograph speed to allow two or even three seconds per tube. It has been the usual procedure to take samples at about one per second from the 5th to the 15th second and then over two or three second intervals. In cases of congestive failure, the sampling should extend over a period of 90 seconds. In these cases we have been guided by the circulation time, determined by decholin or other suitable substance, just before the dye run. The sampling was begun about ten seconds before the arm-to-tongue time, and four seconds were allowed per sample.

The elegance of the method of sampling is marred by the spilling of a small amount of blood between the tubes. This can be obviated by putting paraffin bridges between the tubes. Loss of a drop or two of blood is of no practical significance because the lost blood has the same dye concentration as the mean of the two neighboring tubes.

The kymograph used in the Bellevue series was a modified Harvard type with a manually operated escapement mechanism, by means of which intermittent motion of the drum was obtained. The frequency of the intermittent motion was controlled by the speed with which an escapement pawl was rotated, and the amount of the motion was determined by the distance between brass pegs brazed on the base of the drum. This distance was so chosen that the sampling tubes, held snugly together on the circumference of the drum, came successively into filling position with each successive motion of the drum.

With this apparatus, spillage between tubes was minimized and hence for a given rate of sampling a larger volume of blood was collected. The intermittent motion necessitated a timing device which recorded on a separate constant speed kymograph. The moments of sampling were identified by a signal magnet and time lines were marked by a chronograph.

In measuring the cardiac output by the dye injection method, the venous and arterial needles were put in place under novocain and threaded well into the vessels. In the Georgia series an indwelling venous needle was not used, the dye injection being made through the catheter and promptly flushed into the circulation by 15 cc. of saline. A 15 cc. sample, usually arterial, was taken into a heparinized syringe and set aside to make standards and blanks. Any last adjustment of the indwelling needle was made to insure free flow of arterial blood. As soon as the flow was well established, the needle was connected by means of a male adapter to an 8 cm. length of #8F catheter tubing whose other end was held by a stationary arm above the sampling tubes, directing the flow of blood vertically into them as they passed under.

³ Purchased from C. H. Stoelting Company, 424 N. Homan Ave., Chicago 24, Illinois.

In the meantime the indwelling venous needle had been cleared, and with the help of an assistant the arm was raised until the elbow was directly above the shoulder. This position insures delivery of the injected dye into the general circulation as quickly as possible. The arterial samples were taken, as outlined above, after the injection was made.

The size of the dye injection required depends upon the type of colorimeter used. If the Duboscque visual colorimeter is used it is necessary, in order to get readable optical densities in the diluted sample, to inject 250-500 mgm. of dye. The use of brilliant vital red and the preparation of samples for reading by alcohol precipitation of serum proteins has been described elsewhere (9, 10).

It is possible to read much lower values of optical density by various photoelectric colorimeters. Most models have very large cuvettes (one cc. or more), and consequently the small samples yielded by the injection method must be greatly diluted. Nevertheless, it is possible to make accurate determinations by means of the dye injection technique using these instruments when 50-100 mgm. of dye are injected.

With the Beckman spectrophotometer⁴ it is possible to reduce the amount of dye (T-1824) injected to 5 mgm. and to eliminate the making of quantitative dilutions of the serum. This is made possible by the use of microcells⁵ which have a 10 mm. light path and require as little as 0.05 ml. of fluid. Their use has been described by Lowry and Bessey (11) and has proved entirely satisfactory in our hands.

After the serial blood samples were taken (about 0.5 cc. each), they were set aside to clot. It is said that the clot retracts from the walls of the tube more readily and hemolysis is avoided if the latter are paraffined. This is a precaution that is not necessary but may be advisable.

While the samples were clotting, the standards were made up. The initial 15 cc. sample drawn for this purpose was well mixed and then carefully pipetted into quantities of 2, 3 and 4 cc. To each was added, by means of a red blood cell pipette, an accurately measured quantity (approximately 0.004 cc.) of the original dye solution. These were then centrifuged, and the optical densities of the plasma were read at wave length 625 $m\mu$ against the plasma blank. The plot of optical density against concentration in mgm/l. should be a straight line passing through the origin. If this is the case, the relation between optical density and concentration of dye can be set up on a slide rule, and values of optical density transposed to mgm/l. This linear relationship between dye concentration and optical density obtains with such regularity that we see no contraindication to using a single standard sample, though it is important to establish the validity of this short cut before using it. It is important that the optical density of none of the samples reach values in excess of 1.6, where we have found a failure of the linear relationship between optical density and dye concentration. If the injection is held to 5 mgm. or less, several successive measurements on the same individual can be made without encountering this difficulty.

⁴The Beckman Spectrophotometer, Model DU, employed at Georgia, was purchased through a grant to Dr. V. P. Sydenstricker from the Division of Grants, National Institute of Health, U. S. Public Health Service.

⁵Purchased from the Pyrocell Manufacturing Company, 207 E. 84th St., New York, N. Y.

After the serial samples had clotted, and the clots were well retracted, the tubes were centrifuged. At least 0.05 cc. of serum was transferred to the microcuvettes by means of a capillary pipette. Readings were made against a serum blank at wave length $625\text{ m}\mu$. If several determinations are to be made in one day, it is well to have two carriages and eight cuvettes, so that one set may be filled while the other is being read.

Occasional samples may be turbid or hemolyzed. This can be recognized by inspection of the cuvettes and by the fact that the readings are not congruent with the general pattern of the dye concentration curve or the optical density plot of the standard. Turbidity may be roughly corrected for by measuring the optical density at $725\text{ m}\mu$, where the dye has little absorption, and subtracting this value from the

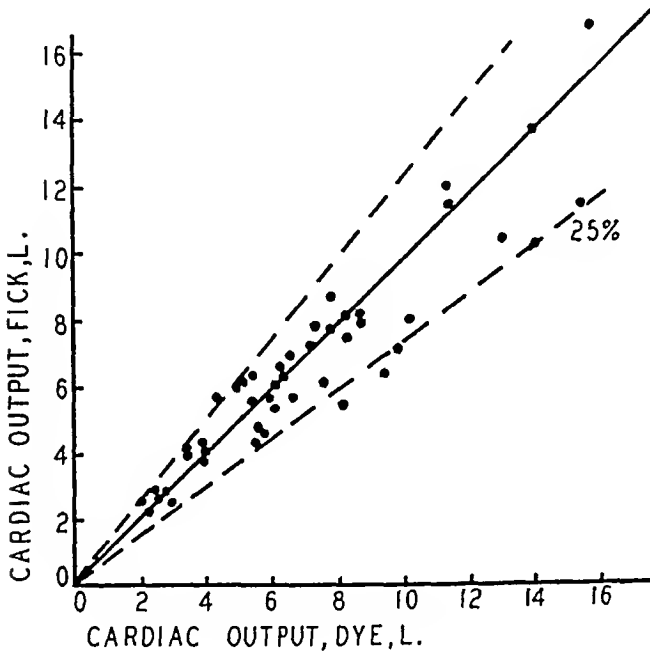


Fig. 1. COMPARISON BETWEEN CARDIAC OUTPUTS measured by the Fick and by the dye injection procedures.

initial reading. Hemolysis can only be corrected for if the relative optical densities of the hemoglobin actually present at wave lengths such as 540 and 625 are known. These corrections and their difficulties have been discussed both for the photoelectric colorimeter and the spectrophotometer (12, 13, 14).

The time of the center of each sample was taken from the record on the smoked drum, and the concentration of each successive sample was plotted against its time. A line connecting these points gives a time concentration curve (fig. 1). When plotted on semilogarithmic paper (K&E #358-63), the points of the descending limb of the curve form a straight line. Depending upon the rate of sampling and upon the speed of the circulation of the subject, there are usually from three to seven points on this straight line. If this is not so, there is no clear evidence as to when recirculation may have occurred, and the experiment must be discarded. If a straight line and clear evidence of recirculation are present, there is no difficulty as indicated above in separating once-circulating dye from that on its second circulation.

In calculating the average concentration, we find it convenient to read concentrations at regular intervals from the smoothed curve rather than to average the concentrations of the actual samples. The concentrations are read every second from the beginning of the curve to the start of the straight line and summated (S_1). A separate sum of similar readings (S_2) is made over the course of the straight line across the first cycle of the paper, e.g., from 35 to 3.5 mgm./l. (see fig. 1). Since the line crosses the next cycle (3.5 to 0.35 mgm./l.) with unchanging slope, the summed points (S_3) over this cycle will be one-tenth of these over the first cycle, or S_2 . If it is necessary to go further, the summed points of the third and later cycles (from 0.6 to 0.06, etc.) can be added in the same manner (S_4 , S_5 , etc.). The sum of all the concentration readings, S , is the sum of S_1 , S_2 , S_3 , etc., and this divided by their number gives the average concentration ($c = \frac{S}{n}$). Since in the above procedure, the number of points (n) is numerically equal to the number of seconds, $S = cT$ and the basic formula of $F = \frac{60I}{cT}$ can be shortened to $F = \frac{60I}{S}$, where F is the flow in l/min.

If the linear relationship between optical density and dye concentration is known to hold under the experimental conditions, it is convenient to obtain average optical density of the dye curve by plotting optical density directly and treating the figures read from this curve as outlined above. The average optical density is then converted to concentration in mgm./l and the calculation proceeds as above.

It is also possible to measure the flow of plasma by using the optical density of dye diluted in plasma rather than in blood. This can be transformed into bloodflow by multiplying by the $\frac{\text{blood}}{\text{plasma}}$ ratio as read from the hematocrit and correcting for trapped plasma (15, 16). An advantage secured by this procedure is that a single standard reading is adequate, as long as the same manufacturers' lot and the same photometer are used.

Whether it is best to use blood-dye standards made up at the time of the experiment or a serum optical density and centrifuge hematocrit is to be decided on the basis of convenience. The $\frac{\text{plasma}}{\text{blood}}$ volume ratio is implicit in both methods. It is determined directly and perhaps inadequately (16) by means of the centrifuge or is determined indirectly by dilution of dye by the volume of plasma in the blood standards. Neither method avoids questions which may be raised on the basis of the fact that the $\frac{\text{cell}}{\text{blood}}$ volume ratio is different in small vessels, in large vessels and in shed blood. These questions seem irrelevant to us because we are measuring the flow of blood from the great veins to the great arteries, each of which have the same hematocrit. The fact that cells pass through the lungs at a slightly higher velocity than does the plasma (14) implies that in the blood of the small vessels in the lungs there must be a greater relative plasma volume than in the large vessels. However, the relative volume flow of cells and plasma through the lung bed must be the same, or else either cells or plasma would necessarily accumulate in the lungs.

Comparison of the two methods. The experimental conditions under which the comparisons were made varied widely. Some subjects were basal and some were exercising, either in the recumbent position or on a bicycle ergometer. The oxygen consumption varied from 182 to 1660 cc. per minute and the cardiac output from 2.5 to 16.8 liters per minute. The subjects included normal staff members and patients suffering from various diseases, including congestive failure of the circulation. All patients were given 0.1 to 0.2 grams pentobarbital and 30 mgm. codeine as premedication. The results are given in table 1 and figure 2.

There was considerable scatter between the observations, though with six exceptions the measurements by the injection method were within 25 per cent of the measurements by the Fick method. More important than the scatter is the fact that there is no evidence of a systematic error. As seen in figure 2 the points are symmetrically placed about the line of identity and the two methods give practically the same average answer (6.6 liters per minute Fick and 6.8 liters per minute dye).

The explanation for the scatter must be looked for in two categories: *a*) technical error of each method and *b*) physiological changes in the state of the subject which may have occurred during the time elapsing between the Fick and dye run.

Technical errors of the dye experiment include those involved in volumetric measurements with the syringe and with the micro pipettes. To evaluate these, repeated weighings were made of the contents of the syringe (water) and of the micro pipette (mercury). These both showed less than two per cent variation. The errors of colorimetry are also of the same order. Disallowing the probability of errors cancelling, the summated errors of this type could aggregate six per cent.

It might seem that since each sample represents an average of the various concentrations reached during the time of the sampling, a concentration plot based on these average values might not represent the true course of the actual concentration curve in the circulation itself. If the rising or falling concentration curve is convex to the base line, then the center of each tube will receive a concentration that is slightly greater than the average and a plotting of the time for the middle of the tube against the average concentration will lead to inaccuracy. A similar discrepancy would obtain in case of a concavity in the true concentration curve. That the error involved is a small one is implied by the results of model experiments already cited (4) in which the measured flow was predicted from the dye concentration curve with errors of the order of three to five per cent.

Another source of error is involved in failure to recognize recirculated blood. In case of the low flat curves seen in severe congestive failure or in dogs after heavy doses of epinephrine (17), evidence of recirculation often does not appear until well after the lapse of twice the first dye appearance time. Such curves should be discarded. The Georgia group have several such curves which are awaiting further study. Unrecognized early recirculation will count the same dye twice, will increase the value of *S* and hence lower the cardiac output figure. Curves of this sort have not been included in this series as indicated by the fact that the average dye cardiac output figure is not less than that of the Fick.

Aside from technical errors of the sort mentioned above, the dye method is more apt to reflect physiological variations. The dye is injected all at once and mixes

TABLE I. CARDIAC OUTPUT DETERMINATIONS BY THE FICK AND DYE METHODS
(BELLEVUE SERIES)

EXP.	SEX	AGE	HT.	WT.	DIAGNOSIS	EXP. COND.	O ₂ CONS.	A-V DIFF.	CO FICK	CO DYE	% DIFF.	AT ¹	BL. VOL.	PULSE PER MIN.
			cm.	Kgm.				vol. %				sec.	L.	
1	F	26	162	50	Bronchiec- tasis rt. pneumo- nectomy.	Rest	191	4.1	4.7	5.5	17	7	3.2	117
2	M	40	165	59	Chr. pulm. emphyse- ma, fibrosis	Rest	235	4.1	5.7	6.5	14	7	4.5	83
3	M	74	164	54	Chr. pulm. emphyse- ma, fibrosis, arterioscler.	Rest	182	4.1	4.4	5.4	23	9	3.7	63
4	M	43	165	60	Bronchiec- tasis	Rest	342	4.4	7.8	8.5	9	6	4.8	129
5	F	34	160	66	Tbc. lft. fibrothorax	Rest	220	3.5	6.3	9.2	46	5	3.2	108
6						Rest	243	3.1	7.8	7.2	8	6	3.6	111
7						Exer.	794	9.1	8.7	7.6	13	2	3.2	173
8	F	39	160	49	Chr. pulm. emphyse- ma, fibrosis	Rest	214	3.1	6.9	6.4	7	5	4.3	93
9						Rest	210	3.2	6.6	6.2	6	7	4.3	93
10	M	43	155	35	Chr. bull. emphyse- ma, congest, fail.	Rest	172	6.8*	2.5	2.0	20	27	3.0	117
11						Rest	182	7.0*	2.6	2.7	4	20	3.1	117
12						Rest	176	7.0*	2.5	2.9	16	20	3.1	119
13	M	49	165	57	Chr. tbc. lobectomy	Rest	232	4.3	5.4	6.0	11	9	5.2	86
14	M	36	176	63	Ess'n. nor- mal	Rest	196	4.2	4.7	5.6	19	15	4.8	47
15						Lt. Ex.	768	7.6	10.1	13.6	35	10	4.8	77
16						Hv. Ex.	1232	9.2	13.4	13.4	0	9	4.8	105
17	M	32	178	67	Ess'n nor- mal	Rest	268	3.8	7.1	9.6	35	9	5.1	91
18						Lt. Ex.	932	8.2	11.4	15.1	32	9	5.1	127
19						Hv. Ex.	1660	9.9	16.8	15.3	9	6	5.1	151

TABLE 1—Continued

EXP.	SEX	AGE	HT.	WT.	DIAGNOSIS	EXP. COND.	O ₂ CONS.	A-V DIFF.	CO FIB	CO DYE	% DIFF.	AT ¹	BL. VOL.	PULSE PER MIN.
			cm.	Kgm.				vol. %				sec.	L.	
20	M	26	178	71	Ess'n normal	Rest	225	3.6	6.2	6.2	0	16	5.7	73
21						Lt. Ex.	505	6.2	8.2	8.4	2	9	5.7	90
22	M	39	181	58	Chr. alcoholism	Rest	213	3.7*	5.8	—	—	—	—	82
23						Rest	269	4.8*	5.6	5.3	5	13	4.4	85
24						Rest	260	4.3*	6.0	4.8	20	11	4.4	93
25						Rest	—	—	—	5.7	—	12	4.3	89
26	M	54	167	53	Mult. lung abscess. convalescing	Rest	229	3.0	7.6	7.5	1	7	—	86
27						Rest	203	2.8	7.2	7.1	1	8	—	81
28						Rest	230	3.8	6.1	7.4	21	8	—	82
29	M	31	159	84	Lung abscess, post lobectomy	Rest	349	2.9	12.0	11.1	8	8	4.7	114
30						Lt. Ex.	676	5.9	11.4	11.2	2	9	5.2	116

Georgia Series

31	M	45	166	55	Congest. fail.	Rest	278	4.8*	4.4	3.8	—14	7	5.2	95
32	M	49	160	66	Congest. fail.	Rest	377	7.0*	5.4	7.9	47	12	6.4	100
33	F	56	165	56	Parox. tachycardia	Rest	251	4.0*	6.3	6.3	0	8	4.7	88
34	F	35	152	55	Hypertension	Rest	303	4.9**	6.2	5.0	—19	15	6.0	81
35	F	34	167	59	Lues, no cardiovasc. involv.	Rest	280	3.5**	8.0	10.0	24	7	6.3	107
36	M	54	160	51	Early congest.	Rest	252	4.0*	6.3	5.3	—15	10	6.0	67
37	M	45	173	63	Ess'n normal	Rest	265	3.4*	7.7	7.7	0	7	5.6	90
38	M	32	178	69	Malig. hypertension	Rest	213	2.8*	7.5	8.1	8	8	6.2	111

TABLE 1—*Concluded*

EXP.	SEX	AGE	HT.	Wt.	DIAGNOSIS	EXP. COND.	O ₂ CONS.	A-V DIFF.	CO FICK	CO DYE	% DIFF.	AT ¹	BL. VOL.	PULSE PER MIN.
			cm.	Kgm.				vol. %				sec.	L.	
39	M	55	163	59	Compens. congest. fail.	Rest	186	4.4*	4.2	3.6	-14	15	5.7	51
40	M	69	158	45	Congest. fail.	Rest	190	3.4*	5.7	5.6	-1	10	4.5	78
41	M	43	168	77	Compens. congest. fail.	Rest	246	5.2*	4.1	3.9	-5	14	5.2	93
42	F	56	163	64	Congest. fail.	Rest	229	10.4*	2.2	2.2	0	17	6.7	100
43	F	40	160	55	Hypertension	Rest	223	2.2*	10.4	12.8	23	7	3.5	103
44	M	69	172	57	Ess'n normal	Rest	203	5.4*	3.7	3.8	2	10	4.7	68
45						Rest	217	3.8*	5.7	4.2	-26	10	4.7	56
46	M	80	175	65	Congest. fail.	Rest	226	5.5*	4.1	3.1	-24	16	—	72
47	M	71	—	—	Congest. fail.	Rest	216	8.0*	2.6	2.5	-4	26	6.9	58
48	M	50	—	—	Emphysema	Rest	272	9.4*	2.9	2.4	-21	26	—	78
Average:									6.6	6.8	13.8			

All mixed venous blood samples were taken from the pulmonary artery except those marked*, which were from the outflow tract of the right ventricle, and **, from the right auricle.

¹ Appearance time of dye.

with the blood stream immediately. The curve reflects the circulation rate over a short elapsed time. The Fick samples, taken over a longer period of time, tend to average out fluctuations of short duration (respiratory). In this study it was impossible to make strictly simultaneous comparisons of the dye method and the Fick method. Although one followed the other within a minute or two, the possibility of a physiological change in cardiac output between the two determinations cannot be denied. Experience in the Bellevue Laboratory indicates that successive determinations by the Fick method check remarkably well. The technical arrange-

ments were somewhat more complicated during the comparative studies, however, and physiological factors on this account may have been greater.

In the case of the Fick procedure, duplicate blood gas analyses are required to check within 0.2 volumes per cent. Because the value for mixed venous blood is subtracted from the value for arterial blood, the errors might be additive and thus amount to 0.4 volumes per cent. If an arterio-venous difference of four volumes per cent is assumed, technical errors in blood gas analysis may result in a ten per cent error in cardiac output determination. Added to this is a small error in the determination of oxygen consumption. The less tangible errors are related to the mixed venous sample and the steady state of the patient. The total error of the

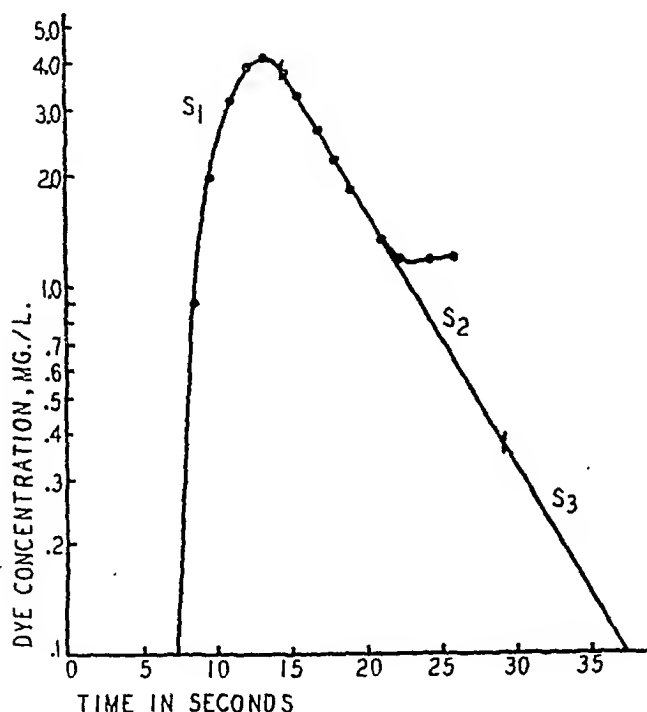


Fig. 2. A REPRESENTATIVE DYE CONCENTRATION CURVE (Case 27). Injected 0.98 cc. of 0.5 per cent T-1824, total = 4.9 mgm. $S_1 = 19.0$ mgm./l.; $S_2 = 20.3$; $S_3 = 2.0$; $S = 41.3$ mgm./l. Cardiac output $= 60 \times \frac{4.9}{41.3} = 7.1$ l/min.

Fick method probably seldom exceeds 15 per cent. In any case, when the estimated errors in the two methods are considered and a possible physiological variable included, a scatter of 25 per cent in the results of the two methods is to be expected.

SUMMARY

The dye injection method and the direct Fick method of measuring the cardiac output were compared in 48 almost simultaneous determinations on 31 different subjects. The results agreed within 25 per cent in all but six determinations. The distribution of results about the line of identity was symmetrical, so that the average of all determinations by one method was almost identical to the average by the other. The scatter was no greater than would be expected when known inaccuracies in both methods are considered.

The data include parallel determinations of cardiac output at rest and during light and heavy exercise in normal individuals, and measurements at rest and occasionally during light exercise in patients suffering from various cardiorespiratory diseases.

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MEASUREMENT OF THE CIRCULATING RED CELL VOLUME WITH METHEMOGLOBIN-TAGGED CELLS

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MALASSEZ (1) appears to have been the first to suggest that the volume of circulating cells might be measured by injecting identifiable foreign cells and noting the ratio in which they appear in the circulating mixture. If identification of the injected cells depends upon differences in their size or shape however, as Malassez pointed out, the ratio is of questionable significance, since such cells may not be distributed within the circulation in the same way as the native erythrocytes. Nizet (2) has recently described a method for labeling red cells visually without deformation, by treating them with phenylhydrazine to produce intracellular Heinz bodies. The method in most frequent current use achieves a non-visual tagging of the red cells by incorporating a radioisotope of iron in the hemoglobin of maturing cells in a donor animal (3). The injected cells are identified in the circulating mixture by their radioactivity.

Erythrocytes may be said to be labeled or tagged if they contain an identifiable substance, differing qualitatively or quantitatively from the constituents of other cells. No exchange of the labeling constituent with other cells or fluids can be permitted to occur if the labeled cells are to be used for an indirect measurement of the circulating cell volume. Cells containing carbon-monoxide hemoglobin, for example, would rapidly lose their identity within the circulation by diffusion of carbon monoxide to plasma, to other red cells, to tissues outside the blood vascular system and to the alveolar air (4). It seems equally doubtful that the isotope P^{32} can be used for tagging red cells, even if it is incorporated within the immature erythrocytes of a donor animal, as described by Hahn and Hevesy (5). Mature erythrocytes have been shown to take up P^{32} rapidly *in vitro* (6, 7). If cells containing this substance are injected into the circulation, it therefore seems probable that some exchange with plasma and with other cells will occur.

Erythrocytes containing abnormal amounts of methemoglobin should, on the other hand, be as effectively labeled, although for a shorter period, as those containing isotope-iron hemoglobin. They lose their methemoglobin slowly by enzymatic reduction of the ferric iron, the process taking place within the red cells themselves (8). In methemoglobinemia, the rate of disappearance of methemoglobin from blood has been reported to be the same *in vivo* and *in vitro* (9). This observation, which is confirmed in the present study, is supporting evidence that reduction within the erythrocytes is the only process which removes intracellular methemoglobin from the circulation and suggests, in addition, that the rate of reduction is relatively uninfluenced by physiological variables. In the present study, suspensions of cells containing methemoglobin were injected into the circulation, and the circulating cell volume was calculated from the methemoglobin content of the circulating mixed blood. If all of the injected methemoglobin is intracellular, the concentration in mixed blood should yield the ratio of injected to native cells.

METHODS AND RESULTS

Methemoglobin was formed in freshly drawn, heparinized dog blood by adding sodium nitrite in the proportion 50-100 mgm. to 100 cc. blood, and allowing to stand

Received for publication May 3, 1948.

for 10 to 15 minutes. Excess nitrite was then removed by adding 5 times the amount of ammonium sulfamate (10). The plasma was removed by centrifuging, and the cells were washed twice by resuspending and centrifuging in their own volume of 0.9 per cent NaCl solution. The white cell layer was drawn off as completely as possible along with the supernatant fluid in this part of the procedure. Enough saline was left on the red cells after the last washing to give a hematocrit in the neighborhood of 75 per cent for the final cell suspension.

Methemoglobin was determined in samples of the cell suspension and of circulating whole blood by the spectrophotometric method of Horecker and Brackett (11) using a Coleman 10-S spectrophotometer at 800 $m\mu$. This method obtains methemoglobin concentration from the decrease in optical density when methemoglobin is converted to cyanmethemoglobin. Since only a ratio of concentrations is required for the present purposes, the optical density values need not be converted into grams of methemoglobin. All samples were immediately pipetted in volumes of 1 cc., after oxalation when necessary, into 10 cc. of an alkaline borate buffer containing saponin and agitated to complete hemolysis. It has been shown that the reduction of methemoglobin is greatly retarded if the cells are hemolyzed in a large volume of diluent, apparently by disorganization of the cellular enzyme systems (12, 13). The methemoglobin content of the hemolyzed samples remained constant, by our methods, for periods of 4 to 5 hours and was, therefore, read as the content at the time of sampling.

The data which are required for the calculation of the circulating cell volume are shown in the general formula: Cell volume = $\frac{Q}{C_m - C_e}$, where Q is the quantity of intracellular methemoglobin injected, C_m is the concentration of intracellular methemoglobin in the circulating blood after the injection and C_e is the concentration in blood before the injection. Since the actual determinations were made on whole blood, the intracellular concentrations required by the formula were obtained from the hematocrits, the concentration of methemoglobin in unit volume of packed cells being obtained by multiplying the whole blood concentration by $\frac{100}{\text{hematocrit}}$. The hematocrits were obtained as the ratio packed cells: total volume in Wintrobe tubes, by methods which have been described previously (14). Justification for converting whole blood to packed-cell methemoglobin is based on consistently negative tests for methemoglobin in the plasma phase both of our injected cell suspensions, and of the circulating blood samples after the injection. The detailed formula is accordingly:

$$\text{Cell volume} = \frac{M_i \times V_i}{\left(M_e \times \frac{100}{Ht_e}\right) - \left(M_o \times \frac{100}{Ht_o}\right)} - V_i \times Ht_i$$

where M_i is the methemoglobin content of 1 cc. of the injected cell suspension, V_i is the volume of suspension injected, M_o is the methemoglobin content of 1 cc. of arterial blood before the injection and Ht_o the arterial hematocrit at that time, M_e is the methemoglobin content of 1 cc. of arterial blood after the injection and Ht_e the arterial hematocrit at that time. The volume of cells injected is obtained from V_i and the hematocrit of the injected suspension, Ht_i , and is subtracted as shown in the

formula. All methemoglobin contents in this formulation are arbitrarily expressed as the optical density values given by 1 cc. in our standard spectrophotometric procedures.

It is not only essential, for our purposes, that the injected methemoglobin be entirely intracellular, as shown by the absence of methemoglobin from the plasma; it is equally important that no additional methemoglobin be formed within the circulation, following injection of the cell suspension, as might occur if excess nitrite were present in the injected material. After treatment with sulfamate and washing as described above, the liquid phase of our cell suspensions consistently gave negative starch-iodide tests for nitrite. The final requirement that the methemoglobin-containing cells be distributed throughout the vascular system in the same manner as normal cells is not capable of direct examination. Hemolysis has only rarely been observed at any stage in the preparation of the cell suspension, and such preparations have been discarded. In a limited number of trials, the packed cell volume has been read at intervals during the processing and has never differed by more than 9 per cent from the initial volume, suggesting that the mean corpuscular volume is not significantly altered. The resistance of the cells to hypotonic hemolysis at the end of the processing has been found to be the same as that of untreated cells from the same source. Figure 1 is a comparison of methemoglobin disappearance rates *in vivo* and *in vitro*. It was obtained by injecting a methemoglobin cell suspension, withdrawing blood after circulatory mixing was complete, and incubating the drawn blood at 38° C. Both the incubated and the circulating blood were sampled at intervals during the following two hours to obtain the curves shown. Such identity of *in vitro* and *in vivo* disappearance rates as is shown in the figure could hardly obtain if there were discriminatory treatment of the injected cells within the circulation.

All the measurements of circulating cell volume in the present study were made on splenectomized dogs in a steady state of barbital anesthesia. The cell suspension, immediately after sampling for its methemoglobin content, was injected as rapidly as possible into a cannulated femoral vein in a volume of 30-70 cc. Time was counted from the end of the injection, which was usually completed within 30 seconds. Arterial samples were drawn from a brachial artery at intervals of one to ten minutes thereafter, by methods which are described elsewhere (14). Figure 2 shows representative curves of methemoglobin disappearance from arterial blood following the injection. In about half the experiments, data resembling the lower curve in the figure were obtained, the rate of disappearance being exponential throughout. In the remainder, an excessive rate of disappearance was recorded for the first two to five minutes, as in the upper curve. During the brief initial phase of rapid disappearance, arterial hematocrits also declined, as happens following the injection of a large volume of a concentrated suspension of normal cells (14). The excessive fall in cellular methemoglobin is accordingly attributed to mixing of the injected cells within the circulation. Alternative explanations seem to be excluded by the data presented below.

Table 1 shows the reliability of the method when it is applied to the measurement of a known volume of drawn whole blood. The accuracy appears from the table to be of the order of 1 to 2 per cent, with no evidence of a systematic error leading to

under- or over-estimation. There appears to be no gain or loss of methemoglobin when the two bloods are mixed *in vitro*, the mixture having the expected mean methemoglobin content. Since studies such as these fail to distinguish intracellular from extracellular methemoglobin, they serve as imperfect controls. Within the circulation any methemoglobin which escapes to the plasma would be expected to leave the circulation quickly, with a resultant lowering of the whole blood methemoglobin concentration and an over-estimation of the circulating cell volume.

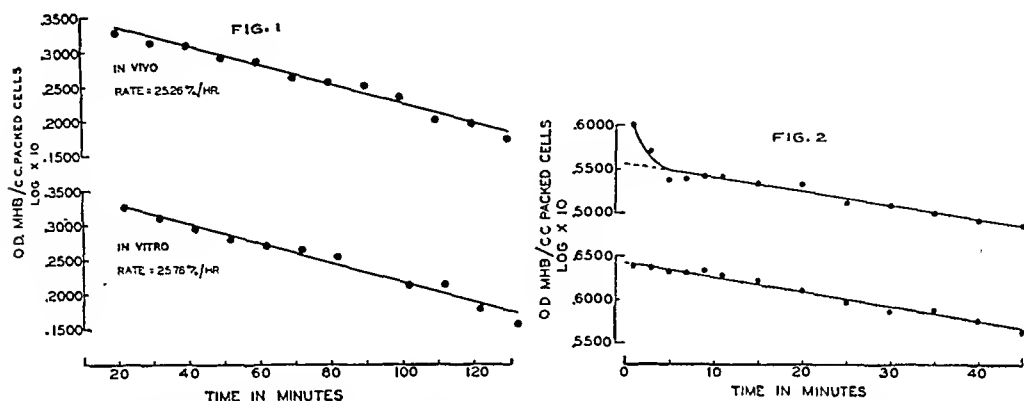


Fig. 1. COMPARISON OF RATE OF DISAPPEARANCE of methemoglobin from the arterial blood of barbitalized dog (*upper curve*) with the rate of disappearance from a sample of the same blood incubated at 38°C. in a flask (*lower curve*). Methemoglobin cell suspension was injected intravenously at time 0, the blood for incubation was drawn at 10 minutes, and both the incubated and the circulating blood were sampled at the times shown. Rate determined by the least squares law. The methemoglobin content of 1 cc. whole blood has been converted to the content of 1 cc. of packed cells by multiplying by 100/hematocrit (see text). Methemoglobin values are stated arbitrarily as optical density readings.

Fig. 2. DISAPPEARANCE OF METHEMOGLOBIN from arterial blood following the intravenous injection of a methemoglobin cell suspension. The upper and the lower curve give the data obtained in two different dogs. The injection of the cell suspension was completed at time 0. Both curves are extrapolated to time 0 as shown, to obtain the methemoglobin concentration used in the calculation of cell volume (see text). Since the cell suspension is sampled just before its injection, all the methemoglobin values used in the calculation are obtained simultaneously. Methemoglobin contents stated as in fig. 1.

Table 2 gives the values obtained for the circulating cell volume in a group of dogs by the present method, and by three other procedures. All the values were obtained within a three-hour period, with the animal in a steady state except as disturbed by the measurements. The volumes shown in the table have been corrected for cells added or withdrawn in the procedures. The three comparative cell volumes were obtained by: *a*) multiplying the arterial cell:plasma ratio by the plasma volume as obtained with the dye T-1824 by the method of Gibson and Evans (15); *b*) multiplying the arterial cell:plasma ratio by the plasma volume as obtained by the 'dye decrement' method recently described (16), and *c*) calculation from the change in arterial hematocrit following injection of plasma or cell concentrates (17). As is shown in the table, the methemoglobin tagged-cell method gave values which were usually fairly close to those calculated by the hematocrit-change method. The 'hematocrit-increase' values in the table were obtained simultaneously with the

TABLE 1. IN VITRO DETERMINATIONS OF A VOLUME OF WHOLE BLOOD BY THE METHEMOGLOBIN METHOD

EXP. NO.	MHb CELL SUSPENSION USED	WHOLE BLOOD MEASURED DIRECTLY	WHOLE BLOOD BY MHb METHOD	ERROR MHb METHOD
	cc.	cc.	cc.	%
1	20	325	324	-0.02
2	20	310	313	1.01
3	10	355	349	-1.70

TABLE 2. COMPARISON OF CIRCULATING CELL VOLUME AS OBTAINED BY THE METHEMOGLOBIN TAGGED CELL METHOD, AND AS CALCULATED BY OTHER METHODS

DOG NO.	MHb	PV _{di} × c/p	PV _{dd} × c/p	HEMATOCRIT CHANGE	
				Increase	Decrease
1	356	448	420	341	361 [†]
2	347	471	385	363	337
3	301	404	323	274	287
4	287	416	391	329	330
5	524	797	613	553	576
6	223	335	313	289	261

Methemoglobin values are given in the column headed 'MHb.' The column headed 'PV_{di} × c/p' gives the values obtained by multiplying the conventional dye-injection plasma volume by the cell:plasma ratio of arterial blood. The next column gives a similar calculation, except that the dye-decrement plasma volume is substituted. The last two columns give the values calculated from the change in arterial hematocrit, "Increase" being the value obtained when the hematocrit was increased by injecting the methemoglobin cell suspension, and "Decrease" when the hematocrit was decreased by injecting plasma. The plasma injection was used for obtaining the latter value, and also for obtaining the dye-decrement plasma volume. All volumes are given in ccm., and are corrected for cells added or withdrawn between measurements. Maximum interval between measurements, 3 hours.

TABLE 3. CELL VOLUMES (CCM.)

DOG NO.	DRAWN (-) OR ADDED (+)	METHEMOGLOBIN METHOD				PV _{di} × c/p METHOD		
		Final circ.	Final circ. corrected	Initial circ.	% change	Final circ. corrected	Initial circ.	% change
7	39	551	512	497	3.0			
8	40	638	598	548	9.0			
9	41	498	457	446	2.4			
10	40	590	550	533	3.1			
11	-307	327	634	648	-2.2	648	719	-9.8
12	-222	158	380	391	-2.8	423	513	-17.6
13	-216	131	347	340	2.0	558	718	-22.3
14	-239	311	550	553	-0.5	566	654	-15.0
15	-216	350	566	531	6.6	633	741	-14.6
16	-420	557	977	1006	-3.0	—	—	—

The total volume of cells added or withdrawn between the two measurements of the circulating cell volume is given in the first column. The next 3 columns give, in sequence, the final circulating cell volume, this value corrected for cells added or withdrawn, and the initial circulating cell volume. The fifth column shows the apparent change in circulating cell volume, as a percentage of the initial value. In the lower group of 6 dogs similar data were also obtained for cell volume as calculated from the conventional dye-injection plasma volume and the arterial hematocrit, and are given in condensed form in the last 3 columns. Values for cells added or withdrawn were obtained from the volume of whole blood and the hematocrit. Interval between initial and final measurement of circulating volumes 1 to 2 hours. The large cell withdrawals in the lower group were obtained by repeated small bleedings with 4% gelatin solution replacement.

tagged-cell values, by injecting a rather large volume of tagged cells and reading both the hematocrit increase and the methemoglobin increase. It has already been pointed out that the hematocrit-change calculation is valid only if the arterial hematocrit has a constant relationship, in a given animal, to the total body hematocrit. Since this assumption does not limit the validity of the tagged-cell calculation, the latter should theoretically give a more reliable value. Trapping or hemolysis of the injected cells, however, should introduce the same error into the tagged-cell and the hematocrit-increase calculation. The hematocrit-decrease values of the table were obtained by injecting plasma. Their reliability is based on the same basic assumption as the hematocrit-increase values, but they cannot be in error because of trapping or hemolysis of injected cells. It is to be observed that in those cases in the table where the methemoglobin tagged-cell value disagrees with the hematocrit-change value, it disagrees almost equally with both the methods of obtaining the latter. This would not be expected if the tagged cells were subjected to abnormal distribution or treatment within the circulation. It seems likely that in these cases both hematocrit-change values are in error because of an error in the assumption of a constant ratio between arterial and total body hematocrit.

Table 3 summarizes the data obtained with repeated applications of the methemoglobin method. In the first group of animals nothing was done between the first and the second measurement of cell volume, except for the injection of cells for the first measurement and the sampling. As shown in the table, when the value obtained on the second measurement, about an hour later, is corrected for these known changes, it is in fairly good agreement with the first. The correction for added cells would have been too large, and the second values correspondingly lower than the first; if the tagged cells injected for the first measurement had been removed from the circulation. No significance is attached, in this small series, to the fact that the second value, with the corrections applied, is slightly larger than the first.

In the second group of animals in table 3, a measured loss of cells was produced by bleeding after the first determination, and the blood was replaced with 4 per cent gelatin solution. When the cell volume found by the methemoglobin method after the hemorrhage was corrected for the volume of cells drawn, it was always in good agreement with the pre-hemorrhage volume. Such agreement was not obtained when the circulating cell volumes before and after the bleeding were calculated from the dye-injection value for plasma volume and the arterial hematocrit. As shown in the table, the cell volume obtained by this calculation was always excessively reduced by bleeding, in agreement with previous reports (18).

DISCUSSION

Systematic errors in an indirect measurement of the circulating cell volume may conceivably be such that:

$$C.V. = M \times a, \quad \text{or, that} \quad C.V. = M + a,$$

where *C.V.* is the true cell volume, *M* is the volume obtained by the method, and *a* is a correction factor. If errors of the first type are present the addition or withdrawal of cells will cause paradoxical changes in the value obtained by remeasurement, unless it happens that an identical error exists in the direct *in vitro* measure-

ment of the volume added or withdrawn. The balance obtained in the present studies when the methemoglobin values for circulating cells are added to direct measurements of the volume bled or injected suggests that the two measurements are of the same order of reliability, so far as errors of the first type are concerned. Both are limited by the accuracy with which the hematocrit reveals the true ratio of cell volume to plasma volume in a sample of whole blood. Even a large error in the hematocrit, if it remained constant, would not disturb the balance in our studies.

None of our data present direct evidence for or against errors of the second type. Systematic errors of this sort seem unlikely, however. In order for them to exist, a certain fixed volume of circulating cells would have to escape detection by the methemoglobin method, if the error is negative; or some of the injected methemoglobin would have to escape into a fixed volume outside the circulating cells, if the error is positive. It is hard to believe that in either event, the volume error would remain constant through the large bleedings and infusions of the present studies.

For the present purposes the validity of the spectrophotometric method for the measurement of methemoglobin does not appear to be an important issue. Arbitrary readings are obtained on blood before and after the injection, and on the injected cells. It does not matter whether the labeling constituent is methemoglobin alone, or whether it is methemoglobin plus some other abnormal substance within the cells, so long as it has the properties disclosed in these studies. The method is simple in application and requires only the usual laboratory facilities. It could probably be simplified still further, since the long series of samples taken after the injection in the present studies is used only to establish a disappearance rate which seems to be the same as in incubated blood. Facilities were not available for comparing directly the methemoglobin method with the radio-iron method. Both methods give values for the circulating cell volume, however, which are consistently less than the values calculated from the hematocrit and the dye-injection plasma volume, the difference being of the same order of magnitude (3, 19).

SUMMARY

The circulating red cell volume was calculated in splenectomized, barbitalized dogs from the methemoglobin content of arterial blood after injecting a suspension of red cells containing large amounts of methemoglobin. Calculations of the cell volume were also made in the same animals from the conventional dye-injection (T-1824) plasma volume and the arterial hematocrit, from the dye-decrement plasma volume and the arterial hematocrit, from the increase in the hematocrit resulting from the injection of the cell suspension, and from the decrease in the hematocrit resulting from plasma injection. The methemoglobin method gave values which were consistently smaller than those calculated from either plasma volume, the dye-injection plasma volume always yielding the largest value. The methemoglobin value was usually fairly close to both the values calculated from the hematocrit change. Both the latter values, i.e., hematocrit decrease with plasma injection and hematocrit increase with cell injection, were in fairly good agreement even when the methemoglobin value was significantly different.

Second measurements with the methemoglobin method after the cell volume had been increased by injection or decreased by hemorrhage (arterial pressure maintained by gelatin infusion in the latter) gave values which agreed satisfactorily with the expected values as obtained from the initial cell volume and the volume added or withdrawn.

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ADAPTATION OF THE TAIL PLETHYSMOGRAPH TO BLOOD PRESSURE MEASUREMENT IN THE MOUSE WITH SOME OBSERVATIONS ON THE EFFECTS OF TEMPERATURE¹

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THE tail plethysmograph method of blood pressure measurement in the intact animal has been subjected to critical evaluation in the case of the rat but not in the mouse. The various factors of importance, such as pressure cuff diameter and length, membrane elasticity and plethysmograph temperature, which can be predicted to influence the resulting readings, have not been evaluated. This report will present some data on the effects of these factors. It may be noted that Kunstman (1), Bonsman (2), Williams, *et al.* (3), Beck *et al.* (4) and McMaster (5) have studied blood pressure in the intact mouse. The last-mentioned author employed a leg pressure cuff, noting the point at which capillary flow in the foot ceased, and checked the values against direct measurement of mean carotid artery pressure. Other workers have not validated their methods by comparison with direct recordings.

METHODS

The apparatus used in this study is shown assembled in figure 1. The apparatus consists essentially of two parts; namely, *a*) an elastic membrane cuff which is in communication with a pressure bulb and a mercury manometer for occluding the blood flow to the tail and for indicating the arterial pressure of the tail and *b*) a plethysmograph for detection of the volume changes of the tail. In addition, there is an animal holder to confine the unanesthetized mouse.

The design of the apparatus is somewhat similar to that described by Byrom and Wilson (6) for the measurement of the blood pressure of the rat, but details have been modified in several respects.

The construction of the pressure cuff is shown in figure 2. It is essentially a thin latex tube which is supported by a brass chamber. It is of disc shape, being 12.5 mm. in diameter and 8 mm. in width. In the center there is 5.2 mm. diameter aperture around which there is a flange of 1 mm. width. The cuff width and diameter can be varied over ± 10 per cent without change in results. A segment of latex tube about 12 mm. long with the diameter of the cuff chamber is passed through the hole and folded over at both ends on the flange and is fixed there by tying with two or three loops of fine strong thread around the recess (see fig. 2C). Into the side of the chamber there is soldered a piece of 1.5 mm. bore brass tube which communicates with the interior space of the chamber, so that when air is forced in, the latex tube is collapsed. Mechanically, the brass chamber is made by soldering two portions as shown diagrammatically in figure 2D. Each portion is prepared by counter-boring and drilling a segment of brass rod. In the smaller portion there is provided a thin shoulder which is just thick enough to center the two portions together for soldering.

Received for publication March 1, 1948.

¹ Aided by a grant from the U. S. Public Health Service.

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Fig. 1. SHOWING THE ASSEMBLY OF APPARATUS used for measuring the blood pressure of the mouse.

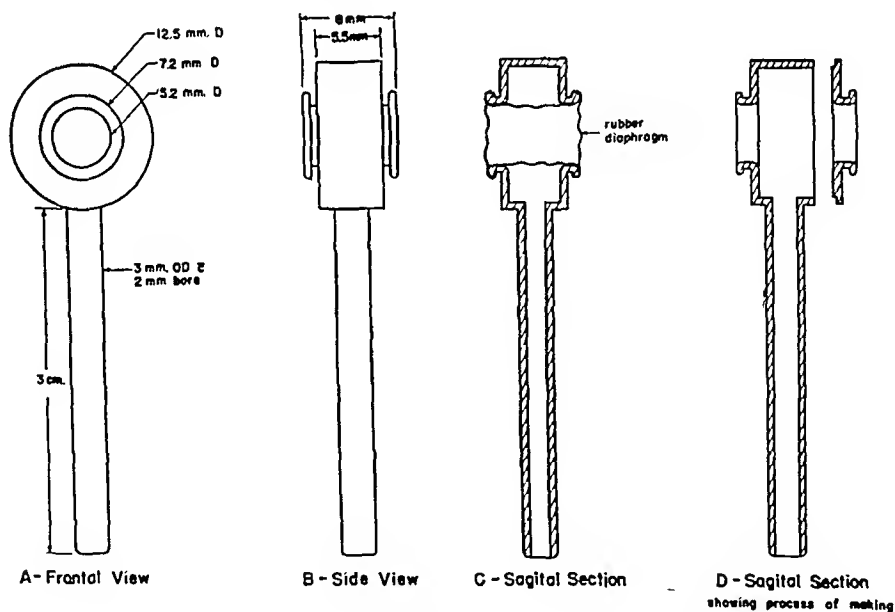


Fig. 2. DIAGRAMS SHOWING THE DIMENSIONS and construction of the pressure cuff for the mouse tail.

The latex³ tube is the critical element of the cuff and should receive special attention. The thickness and elastic properties of the tube are important factors which, among others, determine the accuracy of the method. If the tube is too rigid the higher pressure necessary to close the lumen will obviously result in fictitiously high blood pressure readings. Ideally, the tube should be so distensible that negligible pressure is necessary to cause it to close the space around the tail where the latter is inserted in the cuff. Actually, however, a rubber tube of such properties will not withstand pressures of the order of magnitude of the blood pressure without rupture. In practice it has been found that latex tubes which are totally collapsed in the empty cuff at not more than 50 mm. Hg yield blood pressure values which coincide closely with direct measurements.

The plethysmograph employed consists of two glass tubes. The inner tube is the plethysmograph proper, while the outer one, which covers the greater part of the inner tube, serves as a water jacket (see fig. 1). The plethysmograph proper is about 10 cm. long and about 0.7 cm. in diameter. It is provided with a capillary side tube with a bore of about 0.5 mm. The opening of the plethysmograph is provided with a screw-capped gland made of brass. The hole in the middle of the cap should be just large enough to let the root of the tail pass through. We have prepared several caps with holes of different sizes to fit various sizes of tail. The plethysmograph is filled with distilled water, colored with Congo red. The tail of the mouse is directly immersed in the water. The junction at the gland is sealed with soft surgical soap. Changes in the volume of the tail are indicated by the movement of the meniscus in the capillary tube. The outer water jacket controlling the temperature surrounding the tail has side tubes serving as inlet and outlet, respectively, for circulating water of constant temperature from a thermostat.

The animal holder of the type indicated has proven satisfactory for confining the unanesthetized mouse without struggle. It is made of screen wire mesh moulded and fastened on a wood board and is of such a size and shape as just to fit the head and body with the tail protruding from the holder. A thin sheet of lucite with a slot for the tail is fixed with metal pins against the open end of the animal holder. The head end of the holder is covered by a piece of cotton so as to minimize light stimulation. This latter device is helpful in keeping the animal quiet.

In practice, the mouse is gently put into the holder and allowed to become quiet before use in study. After the cuff is applied to the root of the tail, the remainder of the tail is inserted through the hole in the plethysmograph cap which has already been filled with surgical soap. Finally, the head end of the animal board is tilted up to allow the tail to fall into the plethysmograph, after which the cap is screwed down. It is essential to avoid air bubbles remaining inside the plethysmograph.

Before a blood pressure measurement the rubber tubing between the cuff and the air-reservoir bottle is temporarily clamped, and air is pumped into the bottle to raise the pressure inside to about 50 mm. Hg higher than the predicted systolic blood pressure of the animal. Then the clamp is released to inflate the cuff quickly so as to avoid pooling of venous blood. This sudden inflation of the cuff may give the animal a stimulus and produce some excitement. When the animal has become quiet the pressure of the cuff is lowered until the meniscus in the capillary begins to move

³ The properties vary with different latex preparations. The vultex GL-15-C Moulage from the Latex and General Corporation, Cambridge, Mass., was found to be satisfactory.

outward. The level of the mercury manometer at the moment steady outward movement begins is taken as the blood pressure of the tail artery. Since the movement of the meniscus caused by the moving of the tail or body is not a continuous process, it can be easily distinguished from that produced by the real expansion of the tail volume due to the inflow of the arterial blood. When the pressure of the cuff is lowered further, the outward movement of the capillary meniscus will be followed by a rapid retreat, occurring with resumption of the original tail volume due to escape of venous blood. Three or four successive readings two to five minutes apart are taken.

Comparison of direct and indirect pressure readings. Mice of 28 grams or more were anesthetized with intraperitoneal nembutal, 0.1 mgm. per gram body weight. Heparin in a dose of 1 mgm. was also given intraperitoneally. The carotid artery was cannulated with a 27-gauge needle and connected with a Stratham strain gauge

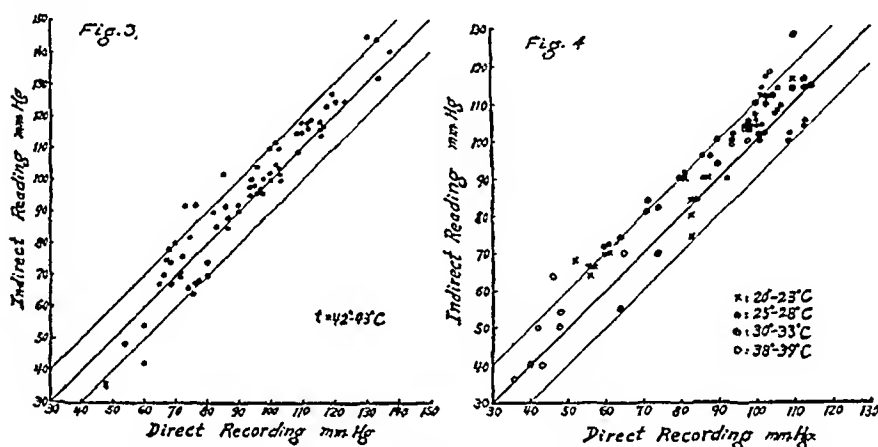


Fig. 3. COMPARISON OF DIRECT and indirect blood pressure readings in the mouse. Tail plethysmograph temperature, 42° to 43°C.

Fig. 4. EXTENT OF VARIATION of simultaneous readings obtained by the indirect and direct methods in mice at temperatures of the plethysmograph below 40°C.

manometer of such sensitivity that 1 mm. scale deflection equalled 2 mm. Hg pressure. This instrument with the connections used does not have a natural free period short enough to record phasic changes in blood pressure. The readings represent mean arterial pressure.

Simultaneous readings of blood pressure by the direct and indirect methods were made on 13 mice, with ten or more observations under various conditions on each. The results are presented in figures 3 and 4. In the former the values obtained with plethysmograph temperatures at 42 to 43°C. are plotted. It is apparent that there is a tendency for the indirect reading to be a little higher, 5 mm. Hg on the average, than the direct reading at pressures above 80 mm. Hg. At lower blood pressures, 45 to 60 mm. Hg, the indirect values tend to be on the low side.

Since in the rat it has been shown (7, 8) that the tail plethysmograph yields fictitiously low blood pressure values unless the temperature is above 42°C. it seemed important to test the question in the mouse. Here, as appears in figure 4, the cor-

responsiveness between direct and indirect measurements is not significantly influenced by temperature between 20° and 39°C. At all temperatures the tendency to a slightly higher indirect than direct pressure reading is seen. It may be noted that the above statements do not imply that tail temperature has no effect on mouse blood pressure, but rather that the tail temperature does not affect the validity of the plethysmograph method of measurement.

Effect of tail temperature on blood pressure. During the course of the above-described experiments it was noted that at high plethysmograph temperatures the blood pressure as measured by both direct and indirect means tended to rise, and to fall when the tail was cooled. An example of this phenomenon is shown in figure 5.

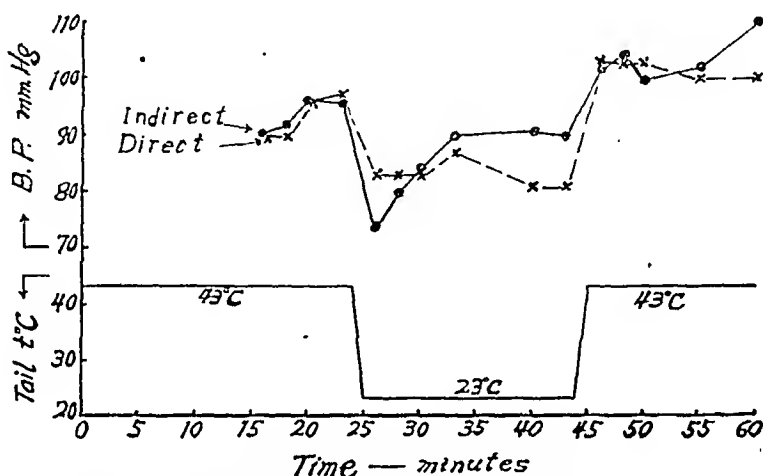


Fig. 5. BLOOD PRESSURE READINGS by indirect and direct measurements in the nembutalized heparinized mouse in relation to tail plethysmograph temperature.

It should be noted that these effects were observed under nembutal, and that the unanesthetized mouse does not tolerate a temperature of 42°C. without struggle.

DISCUSSION

In order to obtain blood pressure measurements in the intact mouse by tail plethysmography it is essential to control the dimensions and elastic characteristics of the occlusion cuff, as has been described above. Values so obtained agree within a few mm. Hg with direct measurements of mean arterial pressure, usually exceeding the latter slightly. A limiting factor in accuracy is the inevitable and slightly variable to-and-fro movement of fluid in the capillary with the respiratory cycle. This prevents accurate detection of the smallest volume change at the height of systole when the cuff pressure is below the systolic pressure but above the mean. It does not, however, prevent making repeated readings which agree very closely. Therefore, the method is accurate for comparative purposes, and especially for measuring changes in blood pressure.

It may be noted that the close agreement between direct and indirect readings at various plethysmograph temperatures is fortunate for certain types of study and is different from the case in the rat. The mouse tail is not covered with as thick a

horny layer as in the rat. This may account for a greater sensitivity of plethysmograph readings in the mouse. On the other hand the tail artery in the rat may constrict at its base at lower temperatures, thus actually lowering the pressure in the shaft.

SUMMARY

1. The details and principles of construction and operation of a tail plethysmograph for blood pressure measurement in the intact mouse have been described.

2. The indirect method of blood pressure measurement has been validated by comparison with simultaneously recorded direct measurements of mean blood pressure over a range of pressures and temperatures in several mice.

3. The tail plethysmograph method of blood pressure measurement in the mouse is not invalidated by working at room temperature. However, the true blood pressure is altered by tail temperature changes in the range between 20° and 43°C.

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PRESSOR SUBSTANCES IN DOG PLASMA INCUBATED WITH RENIN

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THE incubation of a mixture of renin with plasma produces pressor activity. Page (1) and Braun-Menendez and others (2) have separated from such mixtures a heat-stable, dialyzable, alcohol-soluble polypeptide called angiotonin or hypertensin. This is usually believed to be the only pressor product of the reaction between renin and plasma. This belief is the basis of the method of Braun-Menendez (3) for the assay of small quantities of hypertensinogen (renin substrate) in plasma.

The following report re-examines the pressor effects resulting from the reaction between renin and plasma and throws doubt upon the acceptability of the usual simple explanation.

METHOD

Angiotonin was prepared by incubating a small excess of angiotonase-free hog renin with dog plasma at 37°C. for 20 minutes (ca. 0.5 Leloir units (3) renin per cc. plasma). The proteins were removed by precipitation with four volumes of 95 per cent alcohol which was then rapidly evaporated from the acidified filtrate by boiling in an open beaker.

Dog plasma was incubated with the same proportion of hog renin at 37°C. for 20 minutes and the whole mixture injected. The possible pressor action before incubation was controlled by testing the mixture of plasma and renin immediately after mixing.

The tests were made by intravenous injection of from 0.2 to 8.0 cc. of the test material at about 10-minute intervals into a cat, either pithed under ether or anesthetized with sodium pentobarbital. The trachea was cannulated and oxygen re-breathed through soda lime. The blood pressure was recorded from the carotid artery with a membrane manometer. All the data here presented are measurements of diastolic pressure from such a record.

RESULTS

Figure 1 is a kymograph record of such tests. Numbers 13, 14 and 15 show the response to increased doses of angiotonin (1, 2 and 3 cc., respectively). The response to 1.0 cc. was maximal. Further increases were not obtained by increasing the dose. This suggests that in this cat any response greater than 40 mm. Hg (the maximum response to angiotonin) would indicate the presence of a pressor or potentiating agent

Received for publication February 27, 1948.

not present in this preparation of angiotonin (provided there was no change in the sensitivity of the cat).

Numbers 10 and 11 show responses of the cat, at about the same time, to injections of a mixture of fresh dog plasma incubated with hog renin at 37°C. for 20 minutes. The responses were greater than the greatest response obtained by the injection of alcohol-extracted angiotonin made from plasma of the same dog.

Figure 2 is a graph of the data measured from the same experiment as figure 1. Numbers 7, 8, 10 and 11 show responses to injections of incubated plasma; numbers 5, 9, 13, 14 and 15 show responses to angiotonin. The serial numbering indicates

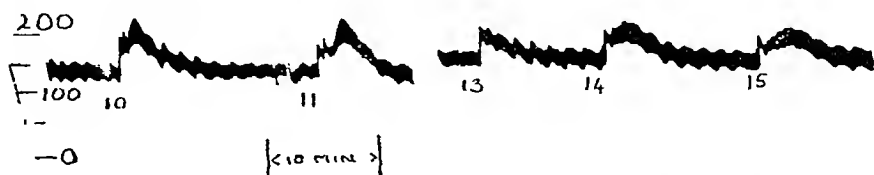


Fig. 1. KYMOGRAPH RECORD showing effect of injections of test material (see text) on carotid blood pressure of anesthetized cat.

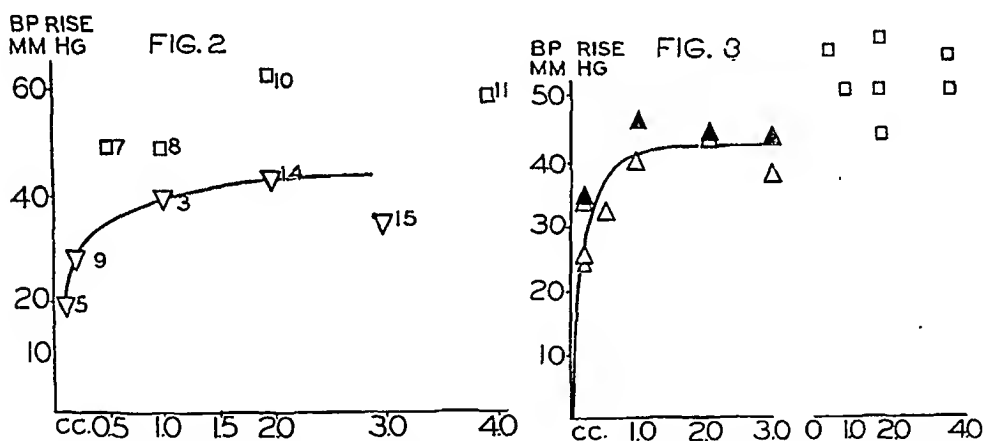


Fig. 2. BLOOD PRESSURE RISE produced in anesthetized cat by injections of angiotonin (triangles) and incubated mixtures of renin and dog plasma (squares). Ordinate: mm. Hg rise of diastolic blood pressure.

Fig. 3. BLOOD PRESSURE RISE produced in anesthetized cat by injections in random order of angiotonin (open and closed triangles represent two different preparations) and incubated mixtures of renin and plasma (squares). Ordinate: mm. Hg rise of diastolic blood pressure.

that the differences in response were not due to a change in the sensitivity of the cat (the question raised in an earlier paragraph).

Figure 3 presents data from 19 injections given to the same cat over a period of five hours. The black line is an exponential curve fitted graphically, passing through 0 and asymptotic to 42, the average of all the points representing doses of angiotonin of 1.0 cc. or greater. Two different preparations of angiotonin were used to show that the phenomenon discussed was not peculiar to one angiotonin preparation. This accounts in part for the scatter of the triangles.

The squares represent injections of incubated plasma and renin and show re-

sponses greater than those obtained from angiotonin. The mean of the six highest points of the angiotonin curve is 42 (maximal response to angiotonin) with a standard error of 1.27; the mean of the six highest plasma points is 53 with a standard error of 1.40. This is a difference of 11 mm. Hg. The standard error of the difference, 1.89 (giving an x/σ of 5.8), may be considered significant since it would rarely occur by chance. (The lowest plasma reading was the last given to this cat. Since it indicated that the animal was becoming insensitive, the experiment was discontinued. However, if this reading is included, the difference between the mean of all seven plasma points and the six angiotonin points is 9 with a $P = < .01$ as indicated by the t test.)

Table 1 shows data from five experiments selected from a large number in which various angiotonin preparations and plasma from many dogs were used. In each of these cases a greater response was obtained from freshly incubated plasma and renin than the greatest response obtainable from any amount of angiotonin.

The values given for maximal pressor responses to angiotonin are smaller than many appearing in the literature. Since the phenomenon here reported concerned

TABLE 1. COMPARISON OF BLOOD PRESSURE RESPONSES

DATE	MAXIMAL DOSES ANGIOTONIN	PLASMA + RENIN (20 MIN.)
2-19-47	24	70
2-21-47	10	58
	10 ¹	
3-26-47	46	58
	43 ¹	
4-10-47	17	22
5- 2-47	31	44

¹ Two different angiotonin preparations.

responses greater than maximal angiotonin responses, it was necessary to confine the work to test animals selected for relative insensitivity to pressor agents.

Whereas the response to our angiotonin reached a maximum as the dosage increased, we did not explore the plateau or maximum response obtainable from incubated plasma and renin in these animals. As the doses increased there was usually a progressive linear increase in response. It was not feasible to explore the range of doses larger than 8.0 cc. in the cat.

CONCLUSION

Since the response obtainable from angiotonin in however great an amount injected can be exceeded by that produced by an injection of incubated plasma and renin, there must be some pressor or potentiating material other than angiotonin in the incubated mixture.

DISCUSSION

The substance which causes the rise or potentiates angiotonin might be a) a product of the reaction of renin with renin substrate, other than angiotonin; b) a product of the reaction of renin with another substrate; c) a product of the reaction

between plasma and something other than renin in the renin preparation. A fourth possibility is that our angiotonin preparations contain some inhibitory substance. The design of the experiments and the timing of the injections were such that an agent in the incubating material potentiating the response to angiotonin would not have been manifest unless it operated only for a very short time after injection. Similarly, if the angiotonin preparation contained an inhibitory substance with any lasting effect, one might expect doses of the incubated material to be less effective shortly after injections of angiotonin than at any other time. Within the limit that none of our injections were given at shorter than five-minute intervals, this was not the case.

In the field of experimental hypertension there is a constant stream of new experiments throwing light on the probable or possible existence of new pressor or vasoconstrictor substances. Of these one may mention the following, which seem to have received more consideration and discussion than others: pepsitensin (4), the persistent pressor substance of Shipley and Helmer (5), the VEM of Shorr (6), the unnamed substance recently described by Dr. Schroeder before the Macy Foundation Conference (7), and the protein vasoconstrictor substance found in hypertension by Page (8).

Before the experiments here reported are allowed to add another to the list of substances whose functions and physiological significance are only partially clear, it is important that the experiments be examined for the possibility that we may be simply presenting one of the previously described substances in a new light. The final decision as to whether this is so can best be made by new experiments designed by those who are intimately familiar with the behavior of these various substances, but an examination of the literature suggests that this pressor manifestation which we are reporting may be due to the substance that Page has reported as a protein vasoconstrictor occurring in hypertension.

The following features lend evidence toward this conclusion. The substance here reported appears only as the result of the reaction between renin and plasma which also produces angiotonin, and it has properties somewhat like those of angiotonin. It would be difficult to demonstrate its existence positively except in circumstances such as have been established by Page. Using the rabbit's ear perfused with Ringer's solution, Page was able to make the test preparation insensitive to angiotonin while it retained its sensitivity to the protein vasoconstrictor substance found in the plasma of hypertensives. As to this substance he states, "... its action is quickly and completely abolished by even low concentrations of alcohol or boiling nor is it ultrafilterable or dializable." Therefore it would not appear in our preparations of angiotonin because of the alcohol and boiling involved, even though it were there in the original incubation from which the angiotonin was made. There is good reason to consider further the possibility of the identity of the agent responsible for the excess pressor response here presented with the vasoconstrictor described by Page.

SUMMARY

Crude angiotonin and freshly incubated renin and plasma mixtures were compared with respect to their capacity to elevate the blood pressure of anesthetized

cats. The freshly incubated mixture in adequate quantities produced greater elevation of diastolic blood pressure than angiotonin, even though an excess or supra-maximal amount was injected. It is concluded that the freshly incubated mixture contains a pressor or potentiating material other than angiotonin.

Acknowledgment is made to Dr. J. Allen Scott for statistical advice and help and to the John and Mary R. Markle Foundation.

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OBSERVATIONS ON THE 'SUSTAINED PRESSOR PRINCIPLE' IN DIFFERENT ANIMAL SPECIES¹

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RECENT studies have demonstrated the presence of a presumably new pressor substance in the blood plasma (or serum) of cats which have been subjected to a moderately prolonged period of hemorrhagic hypotension (1). The pressor substance differs from renin, angiotonin, pepsitensin, epinephrine, pitressin, hydroxytyramine, tyramine and other known biological pressor agents in that it possesses the apparently unique ability to cause a sustained elevation of arterial blood pressure (3 to 4 hours) when injected intravenously into cats which have been nephrectomized 6 to 48 hours before.

The pressor material was not found to be present in the plasma of cats which had been nephrectomized prior to the beginning of the hypotensive state, and it was tentatively concluded that the substance arose from the kidneys possibly as the result of the decreased blood flow (or blood pressure) within these organs. The probability of its having a renal origin was further strengthened by the subsequent finding that similar sustained pressor activity was present in the plasma of cats which were bled a short time following the intravenous injection of semicrude extracts of cat kidneys (2).

The study of the sustained pressor (SP) principle has been extended to include observations involving different animal species. The cat, chicken, dog, hog, horse, human, rabbit, rat and sheep have been used either as test animals or as the source of materials possessing SP activity.

METHODS

Plasmas containing the SP principle were obtained from rats and dogs in the manner previously described for cats (1). Briefly, the animals were anesthetized and by controlled bleeding the mean blood pressure was maintained at 35 to 50 mm. Hg for one to two hours. The blood remaining in the animals was removed, heparinized and centrifuged and the plasma frozen. As parallel experiments to the original observations on cats, normal rats were also given 1 to 2 ml. of an 8 per cent 'DDT' solution intraperitoneally, and immediately following death 12 to 48 hours later each animal's blood was drawn from the heart and the heparinized plasma frozen.

Human blood was withdrawn by intracardiac puncture from 41 patients one-half to one hour post-mortem. To each plasma was added an equal volume of 10 per cent NaCl solution and the mixture adjusted to pH 2.9 with glacial acetic acid.

Received for publication April 12, 1948.

¹A report of this work was presented before the American Physiological Society at the Atlantic City meeting, March, 1948.

The dialyzed supernatant was made 0.6 saturated with ammonium sulfate and of the precipitate formed the water-soluble, nondialyzable fraction was concentrated *in vacuo* to one tenth to one third the original plasma volume and frozen.

For obtaining active plasmas from animals which received semi-crude extract of kidney tissue (2), cats, dogs, rats and rabbits were bilaterally nephrectomized, and from one to three days later the animals were anesthetized with pentobarbital sodium and repeated intravenous injections of the kidney extracts were given over a period of one-half to one hour. Chickens were similarly injected but were not previously nephrectomized because of the technical difficulty in removing the segmented and multivascular kidneys. Each animal received approximately the amount of kidney extract derived from 10 to 15 grams of kidney tissue per kgm. of body weight of the recipient animal. One hour after the last injection of kidney extract each animal was bled and the heparinized plasma frozen.

Nephrectomized cats, rats and dogs, and non-nephrectomized chickens, were used in testing for SP activity of the various plasmas. One to two days following nephrectomy the cats and rats were anesthetized and pithed as previously described (1, 3). The dogs were anesthetized two or three days following nephrectomy, but were not pithed inasmuch as this procedure was poorly tolerated in previous experiments in which an attempt was made to use this type of preparation.

Mean blood pressure in the carotid artery was continuously recorded on smoked paper, using a mercury manometer. Injections were made through a small plastic catheter inserted in the jugular vein.

The plasmas (or sera) to be tested were injected in the arbitrary amounts of 0.5 ml. in the rats (approximately 3 ml/kgm.), 2 ml. in the cats and chickens (approximately 0.7 ml/kgm.) and 5 ml. in the dogs (approximately 1 ml/kgm.).

RESULTS

The blood plasmas from 22 rats which had died as the result of DDT poisoning and from 7 normal rats which had been subjected to hemorrhagic hypotension of one to two hours' duration exhibited SP activity when injected into rats which had been nephrectomized one to two days before (fig. 1). Various viscera were removed from 25 additional rats before subjecting the animals to the period of hypotension. The rats from which the kidneys were removed, with or without the additional removal of the adrenal glands and/or all other abdominal viscera (excepting the liver), yielded inactive plasmas. The rats in which the kidneys were left intact, with or without the removal of the adrenal glands and/or all other viscera (excepting the liver), yielded active plasmas. The effect of hepatectomy alone or in combination with the removal of other visceral organs was not studied.

Almost all of the test rats that had been nephrectomized two days before exhibited sustained responses to the initial injection of active plasma. However, in the animals nephrectomized 1 to 24 hours before and in those in which the renal pedicles were clamped acutely as previously described (4), the initial responses were often unsustained and one or more additional injections of active plasma were necessary before a sustained elevation of blood pressure was observed (table 1). In general, these findings in the rat parallel the previously reported observations on the cat (4).

In both rats and cats the intravenous injection of dibenamine² (20 mgm/kgm.) blocked the pressor effect of epinephrine but did not detectably alter the typical response to the SP principle (fig. 2).

The plasmas of 7 dogs subjected to the hypotensive procedure exhibited SP

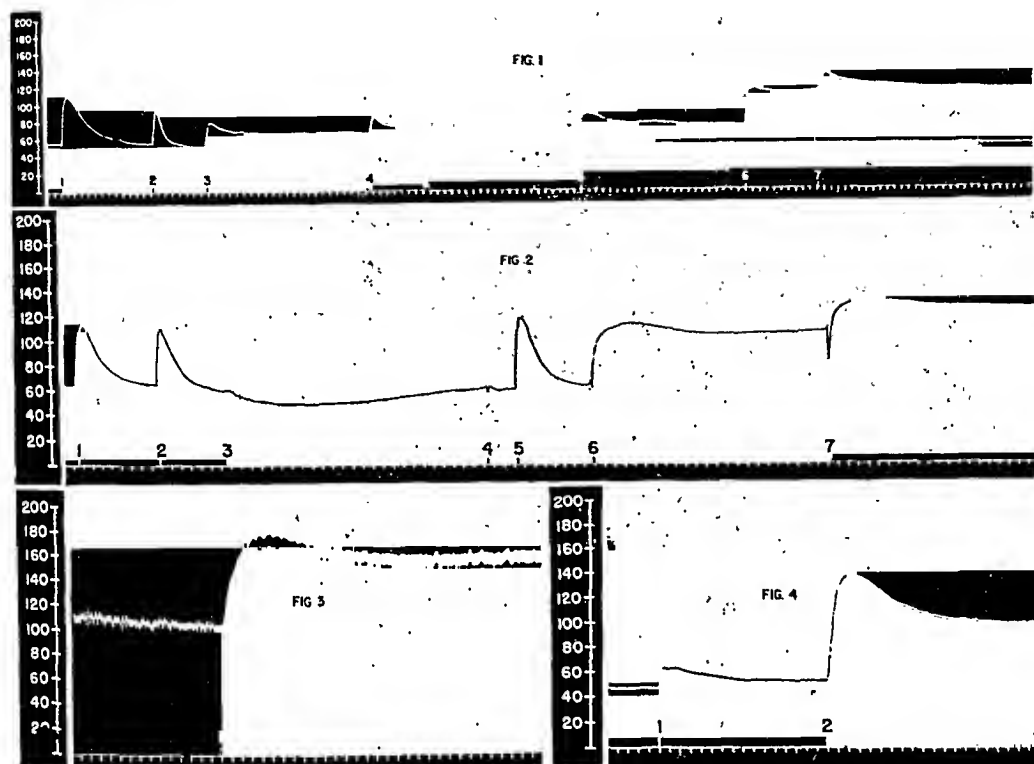


Fig. 1. MEAN BLOOD PRESSURE in carotid artery of 200-gram pithed rat, nephrectomized two days before. I.V. injection: 1, 0.2 unit 'laboratory standard' angiotonin; 2, 0.1 μ gm. epinephrine; 3, 0.5 ml. undialyzed plasma from rat dead from DDT poisoning; 4, 0.5 ml. undialyzed plasma from 'normal' rat (bled under anesthetic); 5, 0.5 ml. of 7% acacia solution; 6, 0.25 ml. undialyzed plasma from cat dead of DDT poisoning; 7, same as 6. Ordinate scale, mm. Hg. Time, 1 minute. (32ZD-12)

Fig. 2. MEAN BLOOD PRESSURE of 230-gram pithed rat, nephrectomized two days before. I.V. injection: 1, 0.2 unit of 'laboratory standard' angiotonin; 2, 0.1 μ gm. epinephrine; 3, 20 mgm/kgm. 'dibenamine'; 4, epinephrine, same as 2; 5, angiotonin, same as 1; 6, 0.5 ml. undialyzed plasma from rat which had received rabbit kidney extract I.V.; 7, 0.5 ml. undialyzed plasma from rat which had received cat kidney extract I.V. Coordinates, same as fig. 1. (32ZD-287)

Fig. 3. MEAN BLOOD PRESSURE of 5.1-kgm. anesthetized dog nephrectomized three days before. I.V. injection: 1, 5.0 ml. undialyzed plasma from rat which had received cat kidney extract I.V. Coordinates same as fig. 1. (32ZB-69)

Fig. 4. MEAN BLOOD PRESSURE of 280-gram rat nephrectomized one day before. I.V. injection: 1, human kidney extract; 2, rat kidney extract. The amounts injected were adjusted so that the human kidney extract contained 60 times as much renin activity as the rat kidney extract as determined by the ability to form angiotonin when incubated with hog serum substrate. Coordinates same as fig. 1. (32ZD-445)

activity when injected into 26 dogs which had been nephrectomized two to three days before. The plasmas from 2 dogs which had undergone a similar period of hypotension (1-2 hours) did not exhibit a detectable degree of SP activity.

² Dibenzy1 β -chloroethylamine.

Of 41 plasmas obtained from human beings post-mortem, 7 possessed SP activity when tested on '2-day nephrectomized' cats. Five of the seven active plasmas were from patients who were known to have gone through a somewhat prolonged period

TABLE I

	PREVIOUSLY NEPHRECTOMIZED				RENAL PEDICLES CLAMPED	NORMAL (NON-NEPHRECTOMIZED)
	2 days	1 day	3-6 hrs.	1-2 hrs.		
Initial pressor response sustained.....	89	50	3	1	0	0
Initial responses unsustained; subsequent responses sustained.....	2	7	4	1	3	0
All responses unsustained						
Hemorrhage, pulmonary edema, very low blood pressure (<40 mm. Hg).....	12	14	5	7	5	1
Animal apparently in good condition.....	8	7	0	6	3	8
TOTAL.....	111	78	12	15	11	9

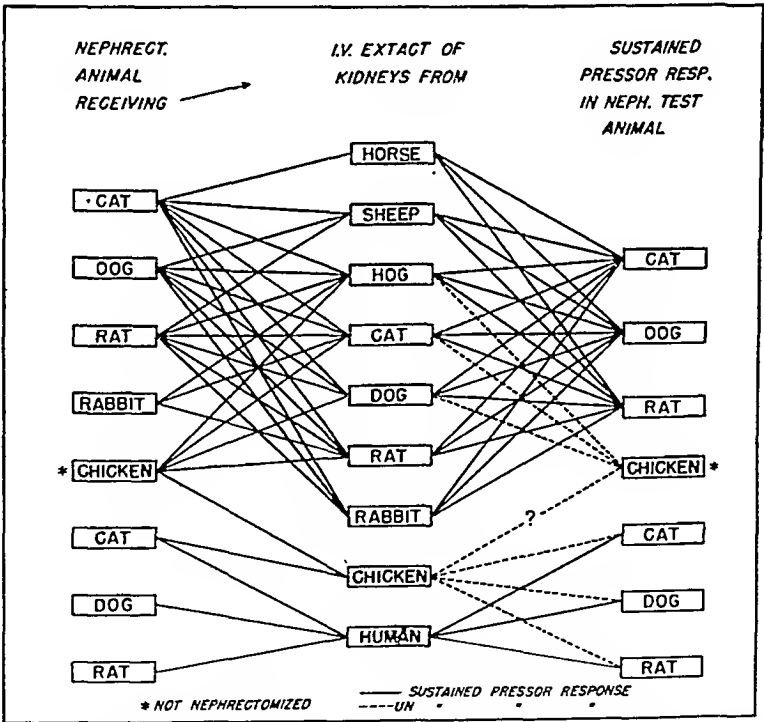


Fig. 5. CHART SHOWING SPECIFICITY CHARACTERISTICS of the sustained pressor principle as indicated by the pressor responses in different species of animals to the intravenous injection of heterologous plasmas from animals which had received I.V. injections of heterologous kidney extracts.

of hypotension preceding death. The clinical diagnoses included myelogenous leukemia, severe burns, strangulated bowel, gunshot wounds (with hemorrhage) and tuberculosis. In two of the patients (undiagnosed coma, malnutrition) adequate data concerning the blood pressure were not available. Records of the patients

from whom the 34 inactive plasmas were obtained indicated that death was more or less sudden and was not preceded by a prolonged state of hypotension.

The nephrectomized cats, dogs, rats, rabbits and non-nephrectomized chickens which had been injected intravenously with extracts of kidneys from the cat, dog, rat, rabbit, horse, sheep, hog and human were bled and the plasmas tested for their ability to cause SP responses in the cat, dog, rat and chicken. In figures 2 and 3 are shown representative pressor responses in the rat and dog. The various combinations tested and the results obtained are summarized in figure 5.

DISCUSSION

The finding that SP activity is present in the plasma of rats, dogs and humans that have undergone a somewhat prolonged period of hypotension parallels the observations made originally in the cat. In the experimental animal, at least, the presence of the kidneys in the animal during the hypotensive state has been found to be necessary for the appearance of the SP principle in the blood stream. Whether or not other organs contribute indirectly to its formation is not known. The gastrointestinal tract, pancreas, spleen and adrenal glands are not directly concerned in the mechanism of formation of the SP principle, since the acute removal of these organs did not prevent the appearance of the material in rats subjected to the shock procedure (with kidneys intact).

Active plasmas were recovered from the blood of the mammals (cat, dog, rat) and the chicken when these animals were injected intravenously with extracts of various mammalian kidneys, but plasmas obtained from mammals or chickens following the injection of chicken kidney extracts containing renin did not cause SP responses in the nephrectomized mammals. The plasmas of both groups caused small unsustained responses in anesthetized non-nephrectomized chickens, but since mammals with intact kidneys have invariably exhibited only unsustained responses (1, 4), no conclusions can be drawn from the latter observations in the chicken.

When chickens were given extracts of cat, dog, hog and rat kidneys intravenously, no rise in blood pressure occurred although the plasmas of these chickens were found to possess SP activity when tested on nephrectomized mammals. The injection of chicken kidney extract into nephrectomized cats did not cause an elevation of blood pressure and their plasmas did not have SP activity when tested on nephrectomized mammals.

These observations suggest that, like renin, the SP principle may be specific with respect to the kidney from which it is derived and the animal in which the characteristic pressor response is observed. There was also found some evidence for the existence of a 'relative' specificity in the response of the nephrectomized rat to the injection of active plasma derived from human sources. In contrast to the cat or dog, the rat exhibited extremely poor sensitivity to the SP principle in the plasmas of patients who had died in shock as well as to the principle in the plasmas from cats and dogs which had been given human kidney extract intravenously. The rat possessed a similar lack of pressor sensitivity to the injection of human kidney extract containing renin³ (fig. 4). However, the blood plasmas from rats which had

³ By incubating human kidney extract and rat plasma *in vitro* it was demonstrated that only a very small quantity of angiotonin was formed.

been given the standard quantity of human kidney extract were quite active when tested on nephrectomized cats and dogs but were very weakly active in nephrectomized rats. From the experiments on the chicken and rat it is concluded that the active pressor principle in the recovered plasma is homologous to the kidney extract injected; the intermediary animal, i.e., the one receiving the kidney extract, appears to play a passive rôle in that it does not alter this specific relationship.

It was suggested in a previous report (2) that when a semicrude kidney extract containing renin is injected intravenously, various components of the extract may be selectively removed *in vivo* by the recipient animal, leaving the SP principle still circulating in the blood stream. This reasoning was prompted by the fact that all but traces of renin⁴ had disappeared from the animal's blood stream while at the same time the plasma had acquired very potent SP activity. When a small quantity of dialyzed kidney extract is injected into a nephrectomized animal the contour of the blood pressure curve suggests that more than one pressor substance may be involved. Characteristically, the blood pressure rises to a rounded peak from which it declines to a new level above the control blood pressure (fig. 4).

Following the injection of dialyzed plasmas containing the SP principle the initial rounded peak is usually small or absent, and the blood pressure gradually rises to the level at which it is sustained. The relatively high concentration of renin in the kidney extract in contrast to the very low concentration of renin in the active plasma makes it seem likely that the initial rounded peak is primarily the response to the renin and the maintenance of the blood pressure elevation is the response to the SP principle. The supposition that the two pressor principles may co-exist in the kidney extract has not yet been substantiated by successful chemical separation or isolation of either principle from crude kidney extract. An alternate theory which is equally tenable would presume that the SP principle does not normally exist as such in the kidney extract, but when the latter is injected intravenously the renin present is partially converted into, or is involved in the formation of, the SP principle. Further work will be required to establish which if either concept is correct.

That the SP principle is relatively stable in the blood stream of nephrectomized animals was originally advanced as a possible explanation for its prolonged action (1). Evidence supporting this view has been obtained from the following experiments. Cats nephrectomized two days before were given the standard quantity of kidney extract intravenously (see METHODS) and following the last injection one to two hours were allowed to elapse before the animals were bled to death. Fifty to 60 ml. of active plasma from each cat was then injected (over a period of 30-45 minutes) into another two-day nephrectomized cat; one hour later the cats were bled. Taking into consideration each recipient cat's original plasma volume (estimated as 4% of body weight) and the quantity of active plasma injected, the approximate factor of dilution was determined. Using comparative tests on nephrectomized cats the concentration of SP activity in the final plasma was not detectably less than could be accounted for on the basis of simple dilution. It is therefore prob-

⁴ The term 'renin' or 'renin activity' as used throughout the paper refers to the specific substance of renal origin which possesses the ability to cause the formation of angiotonin when incubated with homologous blood plasma or serum.

able that the sustained pressor response which follows the injection of an active plasma is due to the continuous circulation of an undiminishing quantity of the principle in the blood stream. These experiments further demonstrate that the pressor principle possesses an unusual degree of stability *in vivo* (in nephrectomized animals) and is not removed from the blood stream in appreciable amounts in one to two hours' time.

SUMMARY

The blood plasmas of rats and dogs which had been subjected to one to two hours of hemorrhagic hypotension were found to contain a pressor principle capable of causing a sustained elevation of blood pressure when injected intravenously into cats, dogs and rats which had been nephrectomized one to three days before. The blood plasmas of human beings who had died following a prolonged period of hypotension possessed similar sustained pressor activity as determined by injection into nephrectomized cats but almost imperceptible activity in nephrectomized rats.

When semicrude extracts of horse, sheep, hog, cat, dog, rat and rabbit kidneys were injected intravenously into nephrectomized cats, dogs, rats and non-nephrectomized chickens the animals' blood plasma acquired the ability to cause a sustained elevation of blood pressure in nephrectomized cats, dogs and rats, but not in non-nephrectomized chickens. The plasmas of chickens and cats which were injected with chicken kidney extracts did not cause sustained pressor responses in the nephrectomized cat, dog or rat. Human kidney extracts, when injected into nephrectomized cats, dogs and rats, caused the appearance in the blood plasma of a sustained pressor principle which was active in cats and dogs but only very slightly active in rats. The specificity characteristics of the sustained pressor principle are similar to those of renin.

The principle may be recovered without appreciable loss from nephrectomized animals which have been given large quantities intravenously one to two hours before. It is concluded that the sustained pressor response which follows the injection of an active plasma is probably due to the continuous circulation of an undiminishing quantity of the sustained pressor principle in the blood stream.

Grateful acknowledgment is made to Messrs. J. H. Tilden, R. J. Parker, C. Wilson and C. L. Goodman for their technical assistance and to the resident medical staff of the Lilly Laboratory for Clinical Research for the procurement of the human blood samples.

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ENZYMATIC CONVERSION OF CYANIDE TO THIOCYANATE

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THE minute amounts of thiocyanate normally present in urine, blood and saliva have stimulated the interest of many workers as to the origin of the cyanide and the pattern of conversion of cyanide to thiocyanate. The problem has toxicologic as well as physiologic importance because of the great toxicity of cyanide. The detoxication of cyanide in the animal body was first demonstrated by S. Lang (1, 2) who was able to show that after the injection of cyanide or of aliphatic nitriles in the rabbit, an increased amount of thiocyanate was excreted in the urine. Similar findings were reported by Heymanns and Mesoin (3). The formation of minute amounts of cyanide from products of protein metabolism and from the nitriles ordinarily present in foods and the conversion of that cyanide to thiocyanate was believed by these authors to account for the thiocyanate normally excreted from the body. The *in vitro* studies of this mechanism were initiated by Pascheles (4), who showed that liver and muscle tissue from the dog were able to produce thiocyanate after digestion with sodium cyanide, liver being more active in this respect than muscle. Kahn (5) concluded from this work and from a series of liver perfusion experiments in which the amount of thiocyanate produced increased with the number of perfusion trips that the liver was an active factor in the production of thiocyanate.

In 1933 Konrad Lang (6, 7) reported his experiments on the *in vitro* production of thiocyanate from cyanide in the presence of sulfur. He postulated that an enzyme was responsible for the conversion of cyanide to thiocyanate, described it as heat-labile and rapidly acting, and gave pH and substrate-concentration optima. The enzyme, which he termed 'rhodanese,' was widely distributed in animal tissues and was present in large amounts in the liver. Lang, therefore, suggested that the formation of thiocyanate was the principal route of detoxication of cyanide in the body and that the liver was the chief site of this detoxication. Cosby and Sumner (8) purified this enzyme to some extent and made further studies of its properties. This paper deals largely with the distribution of the enzyme in various organs and tissues.

EXPERIMENTAL

Tissues obtained from 7 dogs, 5 monkeys, 9 rats and 9 rabbits were assayed for their enzyme content in terms of the ability of one gram of tissue to produce thiocyanate from cyanide. The most extensive work was done on the dog from which tissues of the suprarenals (cortex and medulla separately), liver, kidney, heart, lungs, skeletal muscle, pancreas, spleen, cervical lymph nodes, testes, ovaries, salivary glands, intestine (duodenum, jejunum), spinal cord (cervical, lumbar, sacral regions

Received for publication April 19, 1948.

separately), brain, (cortex, caudate nucleus, a mid-brain section [hypothalamus, thalamus and pons], cerebellum and medulla separately), the optic nerve, the epididymis, thyroid, eye, anterior pituitary and blood components were tested.

The animals were rendered unconscious either by injection of pentobarbital in the case of dogs or by a blow on the head in the case of smaller animals. Monkey tissues were obtained from animals which had received either curare or pentobarbital or both. Tissues were removed from the body as rapidly as possible and homogenates

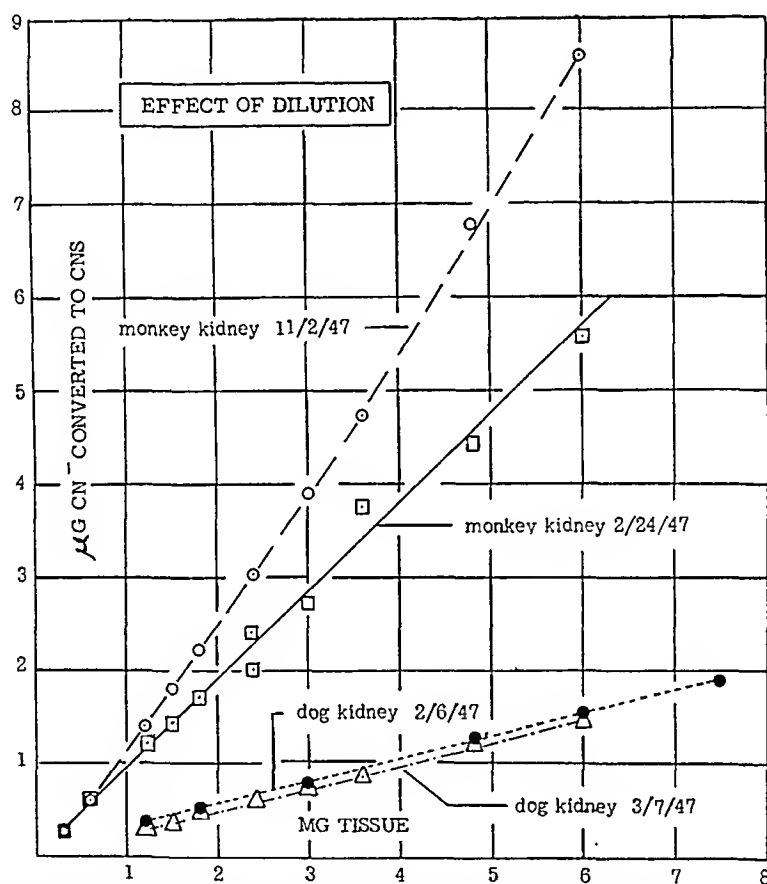


Fig. 1. RELATIONSHIP BETWEEN TISSUE CONCENTRATIONS and thiocyanate formation. *Abscissa:* milligrams tissue in system, *ordinate:* micrograms CN^- converted to CNS^- .

prepared in 10 volumes of distilled water. Tissues weighing 10 grams or more were homogenized in a Waring Blender and filtered through two thicknesses of cheesecloth; those tissues weighing less than 10 grams were homogenized by hand.¹ The homogenates were stored in the refrigerator at 5°C .

Preliminary observations indicated that a system containing phosphate buffer pH 7.4, 0.42 M sodium thiosulfate, 0.3 cc. tissue homogenate, 0.14 M KCN in a total volume of 9.2 cc. added in the order named and shaken for 15 minutes at 37.5°C . gave conditions reasonably near the optimum for all tissues. The enzymatic activity

¹ Ace glass tissue homogenizer.

was stopped by adding 10 cc. of a ferric nitrate-nitric acid solution exactly 15 minutes after the addition of the KCN. The reaction mixture was then diluted to 25 cc. with distilled water, shaken, centrifuged and allowed to stand 15 minutes to permit the violet color formed by the excess thiosulfate to fade. Thiocyanate was determined spectrophotometrically, a standard curve being prepared by adding known amounts of thiocyanate to the tissue system in the absence of cyanide.

Since the color of the iron-thiocyanate complex was too intense at concentrations greater than a few micrograms, it was necessary to dilute tissue homogenates which formed more than this amount of thiocyanate under the standard conditions shown above. The validity of such dilution is shown in figure 1.

Fourteen different sulfur-containing compounds of physiologic and pharmacologic interest were tested for their ability to replace thiosulfate in the standard system. The compounds were added in such amounts that they could furnish the same quantity of sulfur as did the thiosulfate. Slices, minces and homogenates prepared from representative samples of the dog and rabbit liver and of rabbit cerebral hemispheres were compared in respect to ability to form thiocyanate in the standard system.

RESULTS

The dogs studied showed a wide variation in the enzyme content of their tissues (table 1). However, in all dogs irrespective of the absolute value obtained, certain tissues always ranked highest and others always contained minimal amounts of the enzyme. The suprarenal gland had the highest enzyme content, the activity being almost entirely concentrated in the cortex. The dog apparently is unique, as the suprarenals from other species showed no great concentration of the enzyme as compared with other organs. Of the latter, liver was the highest although its value was often as low as a fifth of that of the suprarenals. The various parts of the brain and spinal cord, the kidney and testes had relatively large amounts; other tissues, such as heart, intestine, spleen, lung, muscle and salivary gland, had smaller quantities, while the content of red blood cells and plasma was barely measurable. The two female dogs had the highest concentration of enzyme in the liver; in one, the liver value was even greater than the suprarenal. Unfortunately, not enough females were available to investigate a sex difference.

The monkeys studied contained more enzyme in the liver than in any other organ, the kidney being the next highest. The content of these organs was much higher than in the dog. Heart, and muscle also, contained higher concentrations than the dog tissues. The other organs, and the parts of the central nervous system, however, ranked with the same tissues from the dog.

Rabbit homogenates tended to have a little higher enzyme activity than those of monkey organs. The difference, however, was again not marked in the parts of the central nervous system. The same may be said for the rat—liver and kidney contained higher concentrations of enzyme than were present in the other species, but the parts of the central nervous system were about the same as in the dog.

In general, it can be said that while the enzymatic activities of liver, kidney, muscle and suprarenals vary markedly from species to species, the activity of the

TABLE 1. ENZYMATIC ACTIVITY OF TISSUES OF THE DOG, RHESUS MONKEY, RABBIT, AND RAT
Values are mgm. of —CN converted to —CNS, per gram of tissue

	DOG		RHESUS MONKEY		RABBIT		RAT	
	Range	No. of obs.	Range	No. of obs.	Range	No. of obs.	Range	No. of obs.
	mgm./gm.		mgm./gm.		mgm./gm.		mgm./gm.	
Suprarenals								
whole.....	2.14-3.60 (5.46, 4.50)	6	0.14-1.35	3	1.24- 3.94	2	0.27- 0.41	2
cortex.....	2.86-5.62	2						
medulla.....	0.27-1.12	2						
Liver.....	0.78-1.46 (4.91, 6.28)	7	10.98-15.16 (5.98)	4	7.98-18.92	9	14.24-28.38	9
Brain								
cortex.....	0.34-0.92	7	0.27	1	1.41- 1.44	2	0.70- 0.72	2
caudate nuc.....	0.27-1.06	7	0.34-0.50	2	0.13- 0.18	2		
midbrain.....	0.52-1.35	6	0.22-0.80	2	1.17- 1.39	2	0.73- 1.13	2
cerebellum.....	0.21-1.22	7	0.33	1	0.63- 1.24	2		
medulla.....	0.38-1.52	7	0.49-0.85	2	0.91	1		
Spinal cord								
cervical.....	0.15-1.08	7	0.56-0.57	2	0.89- 0.90	2	0.16- 0.18	2
lumbar.....	0.12-0.84	4	0.20-0.52	2	0.35- 1.74	2	0.23- 0.27	2
sacral.....	0.16-1.41	4	0.23-0.28	2	0.59- 1.10	3	0.56- 0.74	2
Heart.....	0.11-0.14	6	0.48-0.82	3				
Kidney.....	0.42-0.74	6	2.46-3.58	4	6.20- 7.69	3	10.44-11.08	2
Testes.....	0.32-0.41	5	0.38-0.46	3	0.32- 0.36	2	1.24- 1.61	2
Epididymis.....	0.29	1						
Ovaries.....	0.42	1			0.30	1		
Lung.....	0.16-0.17	3	0.11-0.21	2	0.40	1		
Spleen.....	0.10-0.14	2	0.12-0.34	2	0.20	1		
Muscle.....	0.03-0.19	6	0.23-0.57	3	0.18	1		
Intestine								
duodenum.....	0.05-0.11	3						
jejunum.....	0.04	1						
Eye.....	0.02	1						
Optic nerve.....	0.35	1						
Salivary gl., parotid....	0.05-0.36	3	0.99	1				
Lymph node.....	0.08-0.13	2						
Pancreas.....	0.14-0.28	4	0.12-0.44	2				
Thyroid.....	0.05-0.94	3						
Ant. pit.....	0.26	1						
Whole blood.....	0.01-0.02	2						
Erythrocytes.....	0.01-0.02	2						
Plasma.....	<0.01	1						

Figures in parentheses are single observations falling outside the normal range.

parts of the brain does not. The species grouped according to increasing activity of their liver and kidney homogenates are dog, monkey, rabbit and rat.

Table 2 compares the enzymatic activity of slices, mince and homogenates pre-

pared from the same tissue. The minced tissue converted approximately twice as much cyanide as did the sliced, while the homogenate made approximately 5 to 17 and 3 to 8 times as much thiocyanate as did the sliced and minced tissues, respectively.

The ability of other sulfur-containing compounds to replace thiosulfate in the system is negligible. The best replacement was obtained with sulfide, thiourea and

TABLE 2. MGM. —CN CONVERTED TO —CNS BY 100 MGM. TISSUE

	RABBIT CORTEX	RABBIT LIVER	DOG LIVER
Slice.....	7.1	30.5	7.3
Mince.....	12.8	71.7	11.0
Homogenates.....	109.6	514.0	37.0

TABLE 3. REPLACEMENT OF SODIUM THIOSULFATE BY OTHER SULFUR-CONTAINING COMPOUNDS
(Sodium thiosulfate = 100% activity)

COMPOUND	% ACTIVITY OF STANDARD SYSTEM	COMPOUND	% ACTIVITY OF STANDARD SYSTEM
Sodium thiosulfate.....	100	Methionine.....	1
Sodium sulfide.....	4	Cystine.....	1
Sodium tetrathionate.....	spontaneous conversion	Cysteine.....	1
Thiourea.....	4.5	Thiodiglycol.....	0
α -Naphthylthiourea.....	4.6	Diphenylsulfide ²	0
Thiouracil.....	1	Diphenyldisulfide ²	0
Dithiobiuret ¹	1		

¹ Supplied through courtesy of American Cyanamide Company.
² Supplied through courtesy of General Chemical Company.

α -naphthylthiourea, but no conversion occurred with the sulfur-containing amino acids (table 3).

DISCUSSION

The enzyme responsible for the conversion of cyanide to thiocyanate is widely distributed in the animal body and in relatively large amounts. Mendel *et al.* (9) report a different distribution pattern than we do. This discrepancy may be due to the fact that they did not use the same amounts of cyanide and thiosulfate that we did and that they studied only tissues from the rat, whereas most of our data is based on the dog. On the basis of their data they suggest that this enzyme is concentrated in those tissues whose metabolism would be markedly inhibited by small amounts of cyanide, for the purpose of removing any traces of cyanide formed in metabolism. Our distribution data on tissues from dogs and other species do not permit us to concur with their opinion and we feel that an enzyme so widely distributed in such large quantities probably has some metabolic function other than the detoxication of cyanide.

It is difficult to make a comparison between our data and Lang's (6) because he used an acetone powder, the preparation of which may have resulted in variable losses of activity and because he expressed his data in arbitrary units based on the activity of

the dry powder. But, with one or two exceptions, our data on the distribution of activity in tissues of the dog seem to agree well with his. The data suggest that even though the liver undoubtedly plays a major rôle in removing cyanide, detoxication probably occurs in all parts of the body. The high activity of various parts of the brain, taken together with the very large blood flow through that organ and the large mass of muscle even with a relatively low activity, should make brain and muscle important sites of detoxication.

In attempting to relate *in vitro* work to the intact animal that has been poisoned with cyanide, several questions present themselves. An *in vitro* system such as described here completely avoids the problem of cellular permeability. Cyanide is known to enter the cell readily and probably combines at once with the cytochrome oxidase present (10). Thiosulfate on the other hand probably penetrates more slowly, if at all, since Gilman *et al.* (11) have shown that 70 to 80 per cent of injected thiosulfate is excreted unchanged. The data presented on the different activities of slices, mince and homogenate indicate that the factor of permeability may play an important part in determining how much cyanide can be detoxified by an animal, irrespective of the amount of enzyme present.

Calculating from the *in vitro* results, the amounts of cyanide which the tissues of the dog can theoretically convert to thiocyanate in a 15-minute period are enormous. The whole liver of one dog studied could have detoxified 4,015 grams of cyanide and the total skeletal muscle of the same dog, 1,763 grams.

These amazingly large amounts of cyanide with which a tissue can deal *in vitro* as compared with the relatively small size of a fatal dose suggest that availability of sulfur, not quantity of enzyme present, is the limiting factor in the *in vivo* detoxification. This concept is strengthened by the work of Chen, Rose and Clowes, among many others, showing that injection of thiosulfate is capable of increasing the LD_{50} as much as three to four times (12). *In vitro* at least the conversion of cyanide to thiocyanate does not proceed efficiently unless a thiosulfate concentration of at least three times the molar concentration of cyanide is present. That such a concentration of thiosulfate exists normally in the cell is doubtful. Our data do not suggest that any of the tested compounds other than thiosulfate are effective as sulfur donors, thus leaving the question of the source of sulfur in the cells for formation of thiocyanate still unanswered. Tissue homogenates in our procedure did not contain or could not make sufficient thiosulfate, even over a period of 18 hours, to convert a measurable amount of cyanide to thiocyanate. It is probable, however, that our treatment of the homogenate resulted in loss of the enzyme system which Smythe (13), Fromageot (14) and Garabedian (15) have suggested converts the sulfur of amino acids to sulfide and then to thiosulfate, since that system is known to be unstable. Such a formation of thiosulfate, however, would probably proceed too slowly and be too limited by the quantity of sulfur-containing amino acids present to permit rapid detoxication.

Although the content of enzyme of the various species varies greatly in liver, kidney and suprarenal glands, that of the central nervous system does not. These data may account for the fact that the LD_{50} for intravenously injected sodium cyanide is approximately the same for all these species. On the other hand, the differ-

ence in enzyme activity of the livers from two of the species tested appears to have a relation to the rate at which cyanide is detoxified when given in sublethal doses. Mukerji and Smith (16) reported that rabbits were able to detoxify cyanide rapidly—nearly all the cyanide being recovered as thiocyanate in the urine within 24 to 48 hours. In the dog, however, less than 25 per cent of the injected cyanide was recovered within a period of seven days.

SUMMARY

1. The distribution of the enzyme capable of converting cyanide to thiocyanate in homogenates prepared from tissues of the dog, rhesus monkey, rabbit and rat was studied.

2. No marked species difference was observed in the activity of homogenates prepared from parts of the central nervous system. Other tissue homogenates, however, showed a wide variation, the activity increasing in the following order: dog, rhesus monkey, rabbit and rat.

3. The thiosulfate ion was the only sulfur-containing compound found capable of efficiently providing sulfur in the *in vitro* system.

4. Data are presented to show the intracellular character of the enzyme responsible for thiocyanate production. The significance of the enzyme system in connection with the *in vivo* detoxication of cyanide is discussed.

Since this paper was written determinations of the enzyme activity of samples of human liver, kidney and suprarenals obtained at autopsy have been made. These gave values of 1.30, 0.22 and 0.14 mgm. of cyanide converted to thiocyanate for the three tissues respectively.

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EFFECTS OF ANTICHOLINESTERASES ON DIRECT AND INDIRECT STIMULATION OF STRIATED MUSCLE¹

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THE neurone may be expected to exhibit at least four distinct functions, each function being restricted to the part specialized for the purpose: *a*), the maintenance of a generating current by the cell body and its dendrites specialized to function as an electrochemical unit for the production of electrical power; *b*), the generation of nerve impulses by this current as it flows from the dendrites and cell body through the axon hillock membrane back to the external circuit and dendrites; *c*), the conduction of these impulses by the neuraxon proper to the terminal boutons; and *d*), the final activation of the end organ or neurone by the terminal motor end plate or synapse (1, 2). Granting that all of these functions are cholinergic, anticholinesterases may conceivably affect these processes in equal or varying degrees. In the light of Nachmansohn's theory that conduction of the nerve impulse by the neuraxon proper is a cholinergic process which is abolished by anticholinesterases, it seemed advisable to obtain more evidence relating to this point as a further aid to an interpretation of central action of the various anticholinesterases.

PROCEDURES

Among the miscellaneous systems classified as cholinergic (3) striated muscle and its motor nerve seemed to offer convenient opportunities for study.

Of the many compounds attributed to have an anticholinesterase effect the following were chosen for this study: physostigmine sulphate (Merck), prostigmine methylsulphate (Roche), di-isopropyl-fluorophosphate (hereafter referred to as DFP), carbon dioxide and strychnine sulphate. In addition the effects of acetylcholine chloride (Merck) were studied.

Frogs (*Rana pipiens*) weighing 60 to 70 grams were used. The frogs were obtained in small lots and kept at a temperature of 5° C. until immediately before pithing and dissection. One animal was used for each experiment. Amphibian Ringer-Locke solution prepared freshly each day was used as the solvent for the compounds studied. Acidity of the Ringer-Locke solution varied from pH 6.9 to 7.3. The solution containing the reagent was agitated and aerated between series of stimuli.

Break shocks were delivered by a rotating stimulus selector at a rate of ap-

Received for publication March 12, 1948.

¹ Reported before the American Physiological Society, 1947. *Federation Proc.* 6: 156, 1947. These experiments were supported by a grant from the John and Mary R. Markle Foundation.

proximately one per 10 seconds through a Porter inductorium. Series of 10 sub-maximal stimulations were made every 5 minutes.

PROCEDURE 1. *Indirect stimulation of gastrocnemius muscle with nerve trunk exposed to anticholinesterases.* Gastrocnemius muscle nerve preparations weighted with 1 gram were used in this procedure. The nerve trunk was subjected to the anticholinesterases with the aid of a double cylindrical chamber made of lucite. Small holes just large enough to permit the passage of the nerve trunk were drilled in the ends and the partition. Sufficient solution was contained in the chamber proximal to the muscle to completely immerse a section of the nerve trunk when the apparatus was in a horizontal position. The distal stimulating chamber served as a moist chamber. The nerve trunk was threaded through the chamber so that it passed from the proximal solution chamber into the second stimulating chamber in which it lay over two platinum wire electrodes. Thus, the portion of the nerve trunk subjected to the anticholinesterase lay between the part of the nerve trunk stimulated and the muscle. With the nerve trunk in place the hole in the chamber end next to the muscle was sealed with petroleum jelly which prevented leakage of the solution onto the muscle.

PROCEDURE 2. *Indirect stimulation of sartorius muscle exposed to anticholinesterases.* The chamber was modified by removing the top of the solution chamber thus providing a trough in which the muscle and the portion of its motor nerve trunk immediately adjacent lay exposed to the anticholinesterase solutions. The nerve trunk was threaded through the small hole in the partition in the moist chamber and on to the platinum wire electrodes as before. The pelvic end of the muscle was secured by a silk thread to a small hook placed in the partition between the chambers. The distal end of the muscle was attached to the isotonic muscle lever weighted with 0.5 grams by a silk thread which was led out of the trough over a small pulley. This procedure subjected the terminal portion of the nerve trunk, the terminal nerve fibers, the myoneural junctions and the muscle fibers to the anticholinesterase solutions.

PROCEDURE 3. *Direct stimulation of curarized sartorius muscle exposed to anticholinesterases.* The sartorius muscle was stimulated by inserting two fine shellacked platinum wire electrodes into the muscle substance of the pelvic end. These wires were sufficiently long and fine to allow them to move freely with the contracting muscle.

Addition of 0.2 units per cc. of 'Intocostrin' (Squibb) to the Ringer-Locke solution and the anticholinesterase solution was found to be the weakest concentration of curare that would consistently abolish muscle contraction induced by indirect stimulation within 10 minutes. The preparation was exposed to this concentration of curare throughout the entire experiment.

PROCEDURE 4. *Direct stimulation of chronically denervated sartorius muscle exposed to anticholinesterases.* Frogs with denervated sartorius muscles were kept at room temperature for a period of 7 to 10 days to allow maximum nerve degeneration before use (4-6).

All records were made on smoked paper with a slow drum.

A control of two series of stimuli separated by a five-minute interval with the

preparation in Ringer-Locke solution was recorded prior to the exposure to the anticholinesterase solution in every experiment of all four procedures.

RESULTS

PROCEDURE 1. *Indirect stimulation of gastrocnemius muscle with nerve trunk exposed to anticholinesterases.* Figure 1 A is a good example of the results obtained with a 0.005 M. solution of physostigmine. The first two series of twitches, with the motor nerve trunk immersed in Ringer-Locke solution, serve as control. The nerve trunk was then immersed in physostigmine solution for 15 minutes. The amplitude of contraction was markedly diminished. Response to stimulation disappeared after 20 minutes of exposure. Withdrawal of physostigmine and reflooding of nerve trunk with Ringer-Locke solution produced prompt recovery. Variations in degree of diminution of contraction amplitude occurred with individual muscle nerve preparations and concentrations of physostigmine. No significant effects were obtained upon the nerve fiber exposed to physostigmine in concentrations below 0.0015 M.

Prostigmine produced only a slight effect. This is shown in figure 1 B which was obtained by exposing a nerve trunk to 0.02 M. solution of prostigmine. The first two series represent the control with the nerve in Ringer-Locke solution. Immersion in the prostigmine solution diminished the amplitude of contraction over a period of 15 minutes, after which the contraction amplitude leveled off for the remaining 30 minutes of exposure to the prostigmine solution. Recovery was gradual and incomplete on returning the nerve to Ringer-Locke solution. Prostigmine solutions weaker than 0.02 M. produced no effects.

Exposure of the nerve trunk to a 0.01 M. solution of DFP in figure 1 C produced a rapid abolition of contractions from which there was no recovery. In agreement with Finerty (7) it was found that unbuffered DFP solutions in effective concentrations were sufficiently acid (pH 2.3-3.5) to cause speculation that the effect might be due, at least in part, to the acidity. For this reason the effects of DFP solutions buffered with sodium bicarbonate to a pH of 6.9 to 7.4 were studied. It was found that roughly two molecular equivalents of sodium bicarbonate were required to neutralize one molecular equivalent of DFP. The effect of a 0.01 M. solution of DFP buffered to a pH of 7.35 is illustrated in figure 1 D. The only effect observed was a diminution of contraction amplitude, and it can be seen that this effect was much less pronounced than with the unbuffered solution. Reimmersion in Ringer-Locke solution resulted in recovery in some preparations (see fig. 1 D). Buffered or unbuffered solutions of DFP weaker than 0.005 M. concentration produced no effect that could be attributed to the drug.

Strychnine produced a rapid diminution in contraction amplitude which was followed by a prompt recovery upon reimmersion of the nerve in Ringer-Locke solution as illustrated in figure 1 E. Consistent effects of this type were produced by solutions of strychnine in concentrations as low as 0.005 M.

Evidence has been presented that carbon dioxide acts as a physiological anticholinesterase by virtue of the changes in acid-base balance produced (8). Solutions of pure carbon dioxide gas in Ringer-Locke solution were prepared by mixing them volumetrically. Changes of contraction amplitude obtained upon exposure of

the nerve trunk to CO_2 were small and perhaps within the limits of error, yet there were indications in some experiments as illustrated in figure 1 F that a 50-volumes per cent solution of CO_2 produced a slight diminution in impulse conduction. Mixtures of a smaller percentage of carbon dioxide were without apparent effect.

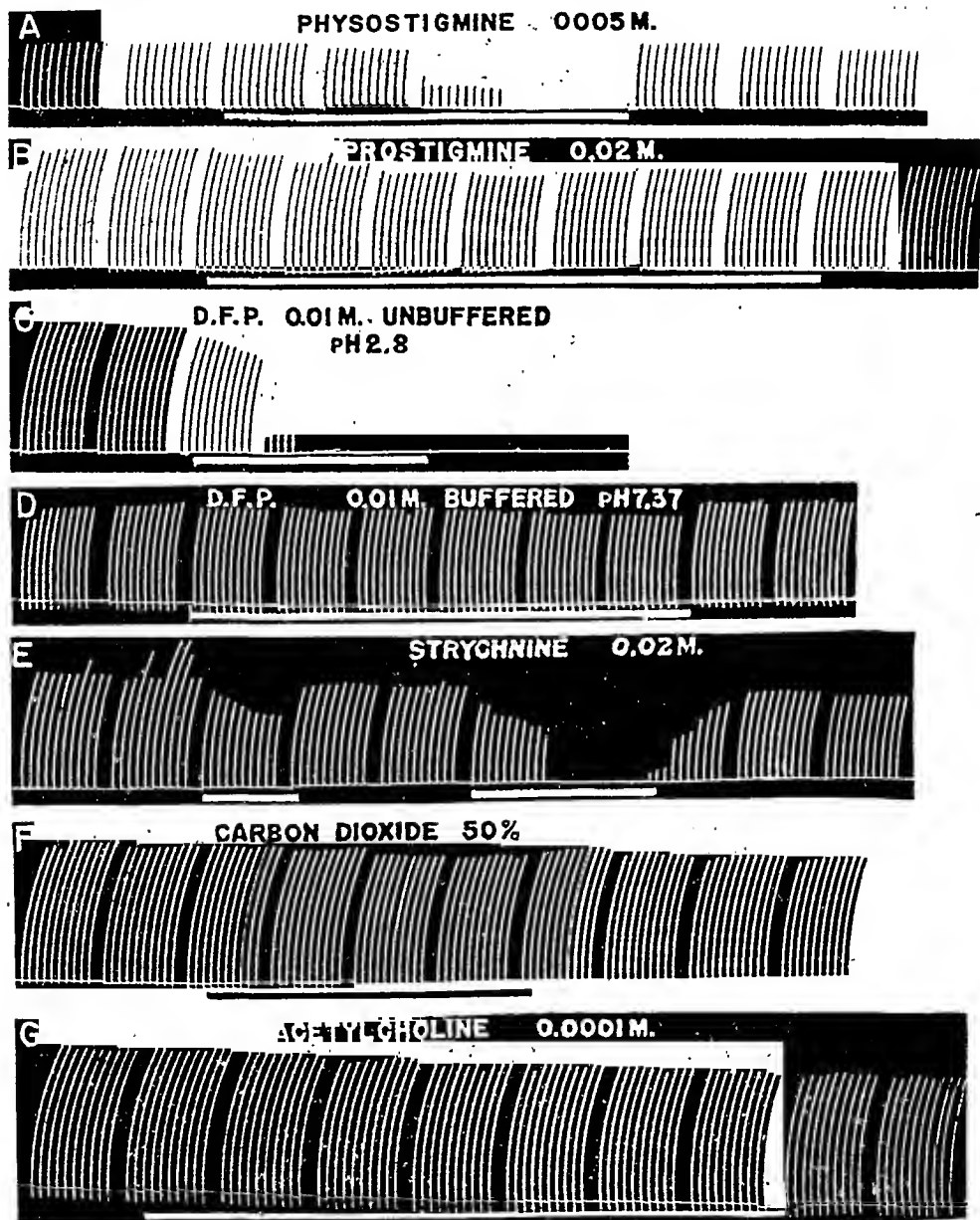


Fig. 1. RESULTS OBTAINED WITH *procedure 1*

Exposure of a segment of the nerve trunk to acetylcholine produced very slight diminution of the response of the muscle to indirect stimulation as illustrated in figure 1 G, where the amplitude of contraction gradually diminished during the 30-minute exposure. There was no significant recovery upon return to Ringer-Locke solution. The changes were perhaps within the limits of error.

PROCEDURE 2. *Indirect stimulation of sartorius muscle exposed to anticholinesterases.* The response of the muscle under these conditions was diminished by every anticholinesterase and to a greater extent than in procedure 1. This is ex-

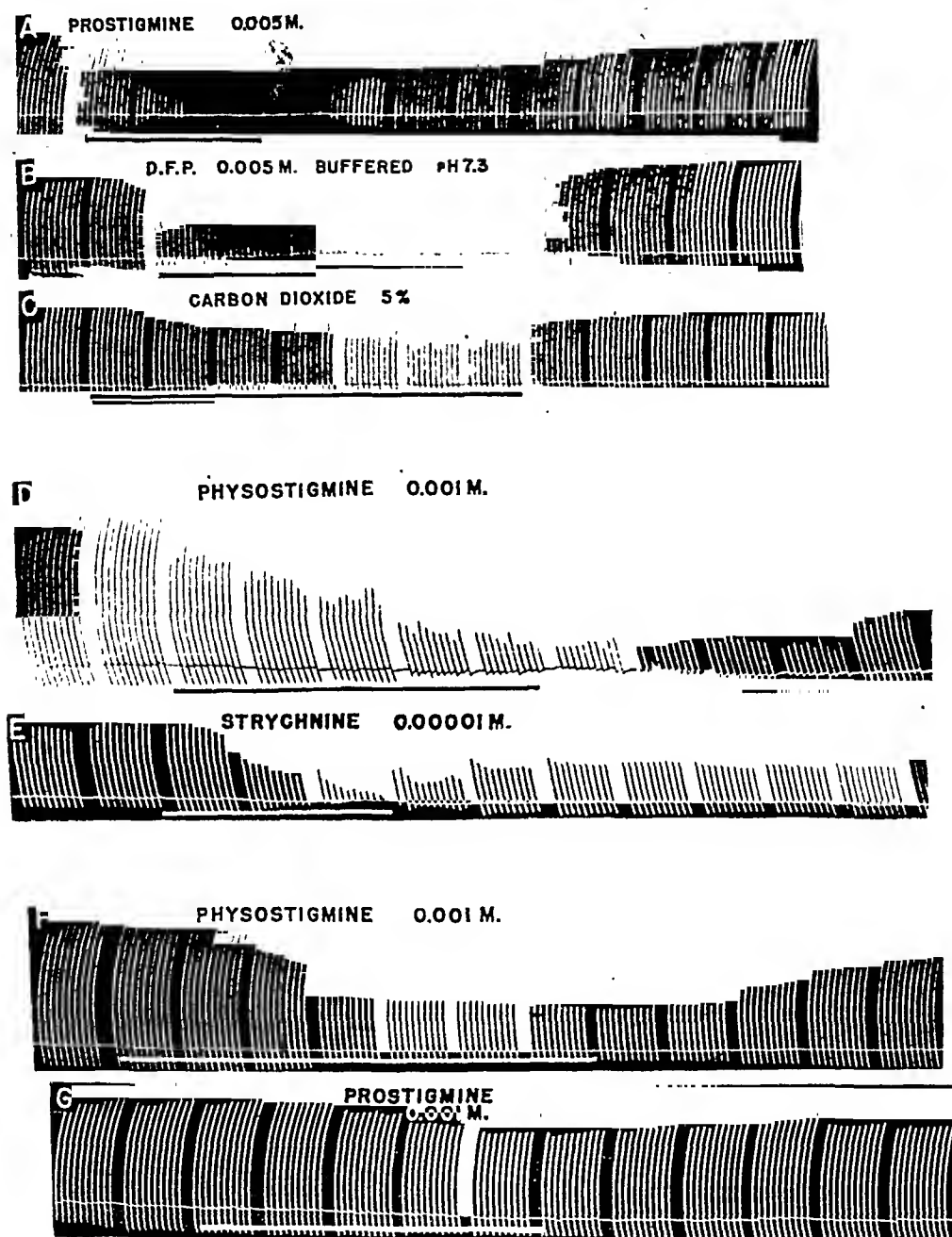


Fig. 2. RESULTS OBTAINED WITH procedures 2, 3, and 4

emplified by the effects of prostigmine, DFP and carbon dioxide in figures 2 A, B, and C.

Prostigmine caused far more profound effects in these preparations than when applied to the nerve trunk only (fig. 2 A). Response to indirect stimulation was

frequently abolished completely. Gradual incomplete recovery was consistently observed after washing and reimmersion in Ringer-Locke solution.

Unbuffered and buffered DFP solutions also diminished the response to indirect stimulation in these preparations in a consistent and positive manner (fig. 2 B). Recovery was more prompt following immersion in buffered DFP and sometimes progressed to a slight increase in contraction amplitude.

This type of preparation also exhibited the greatest sensitivity to carbon dioxide. A diminution of response was obtained in each instance followed by prompt recovery after reimmersion in Ringer-Locke solution. Figure 2 C is an example of the effect obtained with a 5 per cent (by volume) solution.

PROCEDURE 3. *Direct stimulation of curarized sartorius muscle exposed to anticholinesterases.* With the exception of buffered DFP a diminution of response of curarized sartorius muscle preparations to direct stimulation was observed consistently following exposure to solutions of anticholinesterases of sufficient strength. Partial recovery was obtained following washing and reimmersion in Ringer-Locke-curare solution in the majority of instances. The results of this procedure are exemplified by physostigmine and strychnine.

Physostigmine was studied in concentrations ranging from 0.00005 M., which produced no evident effect by a 30-minute exposure, to 0.005 M. which completely abolished response to stimulation after 20-minute exposures. Figure 2 D represents a typical result obtained upon the exposure of the curarized preparation to 0.001 M. physostigmine in Ringer-Locke curare solution.

Strychnine in weak concentrations diminished amplitude of contraction in the curarized preparations as illustrated in figure 2 E. With concentrations of 0.00001 M. and 0.000025 M. a minor transient enhancement of contraction amplitude preceded the diminution. Evidence of recovery appeared immediately after washing and reimmersion in Ringer-Locke curare solution, but complete recovery was not observed.

PROCEDURE 4. *Direct stimulation of chronically denervated sartorius muscle exposed to anticholinesterases.* Exposure of chronically denervated muscle to the various anticholinesterases resulted in a diminution of response to direct stimulation. Effects were not so pronounced as those obtained with preparations having the peripheral nerve mechanism intact as in procedure 2. The results obtained on denervated muscle are exemplified by physostigmine and prostigmine.

Figure 2 F illustrates the reduction of contraction amplitude obtained by exposing a chronically denervated sartorius muscle preparation to 0.001 M. physostigmine in Ringer-Locke solution for a period of 30 minutes. Recovery occurred gradually following return to Ringer-Locke solution and in some instances progressed to completion.

A typical curve demonstrating the effect of 0.001 M. prostigmine solution is illustrated in figure 2 G. There was a gradual diminution of contraction amplitude which was less striking than that produced by anticholinesterases not incorporating a quarternary ammonium group in their chemical structure. Recovery was gradual when obtained and was incomplete.

DISCUSSION

Conduction of the nerve impulse is stated to be a self-propagated disturbance in which the nerve fiber responds to its own action potential as the impulse travels from segment to segment. Exposure to the anticholinesterases of a short stretch of the sciatic nerve lying between the gastrocnemius muscle and the electrodes in procedure 1 consistently diminished the amplitude of contraction produced by uniform stimulation of the unexposed end of the nerve. These results agree with those of Gilman (9) and Nachmansohn (10) and their associates on nerve activity. It was, therefore, concluded that the passage of nerve impulses initiated in the unexposed end of the nerve was blocked in some of the nerve fibers in their passage through the portion exposed to anticholinesterase reagents.

In the light of recent investigations impairment of impulse conduction could be attributed to an increased threshold of stimulation of nerve fiber as demonstrated by Toman, Woodbury and Woodbury (11, 12) or to an altered membrane permeability as proposed by Nachmansohn (10).

The reduced response of a curarized and of a chronically denervated muscle to direct stimulation in curarized and denervated muscle cannot be related to the effects of anticholinesterase reagents on either nerve fiber or motor end plate, and therefore depends upon some change of function of the muscle itself. Impulse conduction in muscle fibers is presumably a similar process to that of impulse conduction in nerve fibers. Thus impairment of impulse conduction in muscle fibers would offer a simple explanation of a diminished contractile response of muscle to direct stimulation since the action potential of a muscle is assumed to stimulate as it advances along the muscle fibers. It is of interest that anticholinesterase reagents may have a depressing action on the processes of impulse conduction in such widely differing structures as nerve and muscle fibers.

In trying to assess the powerfully depressant effects of the anticholinesterase reagents in procedure 2, it must be borne in mind that muscle fibers, motor end plates and the terminal nerve fibers were all exposed to the action of the drugs. The nerve trunk proper, however, was unexposed and consequently it may be assumed that a uniform number of impulses was carried up to the muscle preparation with every induction shock delivered to the nerve. The extremely fine caliber of the terminal nerve fibers as compared with their parent fibers in the nerve trunk as well as the absence of a protective myelin sheath would tend to make them highly susceptible to chemical block. If this assumption is true, great terminal blocking of nerve impulses could be sufficient cause for the great reduction of response of the muscle to indirect stimulation.

It may, therefore, be concluded in agreement with Toman, Woodbury and Woodbury (11) that the excitability of nerve fibers is depressed by anticholinesterase reagents. This conclusion may in its turn throw needed light on the physiology of the neuron and on the nature of the central action of anticholinesterase reagents. The theory of synaptic conduction is deeply involved for this theory holds that a nerve impulse enters the dendrite, that it is then automatically propagated in the direction of the neuraxon where its conduction is continued in the accepted manner

alluded to above. According to this theory, the central nervous system functions as a highly developed *conducting* system of nerve impulses, but if our observations on impulse conduction in nerve fibers and muscle fibers are applicable to the function of the neuron, in accordance with the theory of synaptic conduction of nerve impulses, it would seem to follow that the impaired conduction produced by anticholinesterase reagents must of necessity impair central nervous activity. How then is the central potentiating action of each of the anticholinesterase reagents here studied (13, 14) to be explained? It would seem that these reagents must produce an additional effect which more than compensates for decreased excitability first established by Toman, Woodbury and Woodbury (11). In the light of the theory of neuron function outlined in the introduction of this paper, generation of nerve impulses is the resultant of two factors: the intensity of the neurocellular generating current and the excitability of the rhythmically responding membrane of the axon hillock to this current. Thus, a disproportionate increase of generating current resulting from an acetylcholine sparing action of anticholinesterases as compared with a secondary depression of excitability would increase the rhythmic discharge of the axon hillock. A disproportionate decrease of excitability, however, could depress the activity of the axon hillock which offers a possible explanation of exceptions to the predominantly central potentiating effects of anticholinesterases.

In the light of this discussion, it is concluded that the universal peripheral impairment of conduction of impulses in nerve fibers and muscle fibers as contrasted with the central potentiation of nervous activity by anticholinesterase reagents is incompatible with the theory of synaptic transmission of nerve impulses.

SUMMARY

1. A comparative study of the action of several anticholinesterases on muscle and nerve-muscle preparations of the frog was undertaken to obtain more evidence on the nature of the central action of various anticholinesterases.

2. The following agents known to inhibit cholinesterase were used: physostigmine sulphate, prostigmine methylsulphate, di-isopropyl-fluorophosphate (DFP), carbon dioxide and strychnine sulphate. All were dissolved in Ringer-Locke solution in various concentrations. In addition, the effects of acetylcholine chloride were studied.

3. Four separate procedures were employed with each of the agents in various concentrations. In procedure 1, a segment of the nerve between the point of stimulation and the muscle was poisoned with one of the agents and the muscle stimulated indirectly. In procedure 2, the muscle was stimulated indirectly following exposure of the muscle fibers, motor end plates and terminal nerve fibers to the anticholinesterase solution. In procedure 3, curarized muscles were subjected to the anticholinesterase agents and stimulated directly. In procedure 4, chronically denervated muscles were subjected to each of the anticholinesterases in varying concentrations and stimulated directly.

4. The common result in each procedure with each anticholinesterase was a diminution of response of the muscle to stimulation. Diminished response to stimulation in procedure 1 must be attributed to impairment of impulse conduction

in nerve fibers. The greater diminution of response in procedure 2 may be caused by greater impairment of impulse conduction in the fine terminal nerve fibers. The diminished response in procedure 3 may be the result of impaired impulse conduction in muscle fibers. The possibility that anticholinesterase reagents may have a depressing action on the processes of muscle contraction was not eliminated.

5. Impairment of impulse conduction in nerve and muscle fibers by anticholinesterases is incompatible with the theory of synaptic conduction of nerve impulses because these reagents potentiate rather than depress central nervous activity. Potentiation of central nervous activity by anticholinesterases is, on the other hand, in agreement with an acetylcholine sparing action of these reagents and a consequent intensification of neurocellular electrotonic currents which are believed to generate nerve impulses.

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AN ELECTROCHEMICAL STUDY OF THE SYNOVIALIS IN DOGS¹

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THE results of numerous investigations on the electrolytes of synovial fluid have been reviewed by Bauer, Ropes and Waine (1), who have concluded that diffusible ions are in equilibrium with those of blood serum and tissue fluids. Experimental studies have been mostly applied to the equilibrium of the normal physiological ions. Diffusion rates of crystalloidal ions through the synovialis have not been quantitatively compared. It is one of the purposes of this investigation to apply electrochemical methods to the determination of ionic diffusion. The problem is analogous to determination of ionic transference and mobility in artificial physico-chemical systems, either by measurement of liquid junction potentials or of membrane diffusion potentials. Classical studies of these effects are those of Planch (9), Henderson (2) and Lewis and Sargent (6) on liquid junction potentials, and those of Michaelis (7), and of Sollner, Abrams and Carr (11) on potentials across membranes with selective ion permeability. Determinations of bioelectric effects of ions in tissues have been reported by Höber (3, 4) and numerous others. An extensive review of the subject is included in Höber's monograph (5).

METHODS

There are certain distinct advantages in working with the joint cavity to study bioelectric effects. The cavity is small and easily penetrated with a needle; it can be conveniently filled with the fluid to be studied and fluids are readily replaced almost quantitatively.

As shown in figure 1, the experimental arrangement included two 18- or 19-gauge needles 1.5 inches long, insulated on the outer surfaces with three coats of lacquer, one (N_1 , fig. 1) inserted into the knee joint cavity medial to the patellar ligament, the other (N_2 , fig. 1) inserted subcutaneously over the medial femoral condyle so that the tips were separated by the synovial membrane and intervening fascial layers. The subcutaneous needle (N_2 , fig. 1) was filled with 0.15 M NaCl solution (approximately isotonic) and about 1 cc. injected into the tissues. A syringe barrel containing the same saline solution was mounted in the needle, forming a continuous column of solution from the syringe barrel to the tissues. The electrode permanently mounted in the syringe was the Ag-AgCl type prepared according to the method of Noyes and Ellis (8). An electrode of the same type mounted in 0.15 M NaCl solution was connected by means of a saturated KCl agar bridge to the solution within the joint cavity. The potential difference between the two electrodes as determined frequently should be less than one millivolt. Electromotive force readings were taken with a type

Received for publication March 5, 1948.

¹ The expenses of this investigation were paid in part by the Arthritis Research Project of the University of Illinois. A preliminary report was published in the *Federation Proc.*, Vol. 7, No. 1, March, 1948.

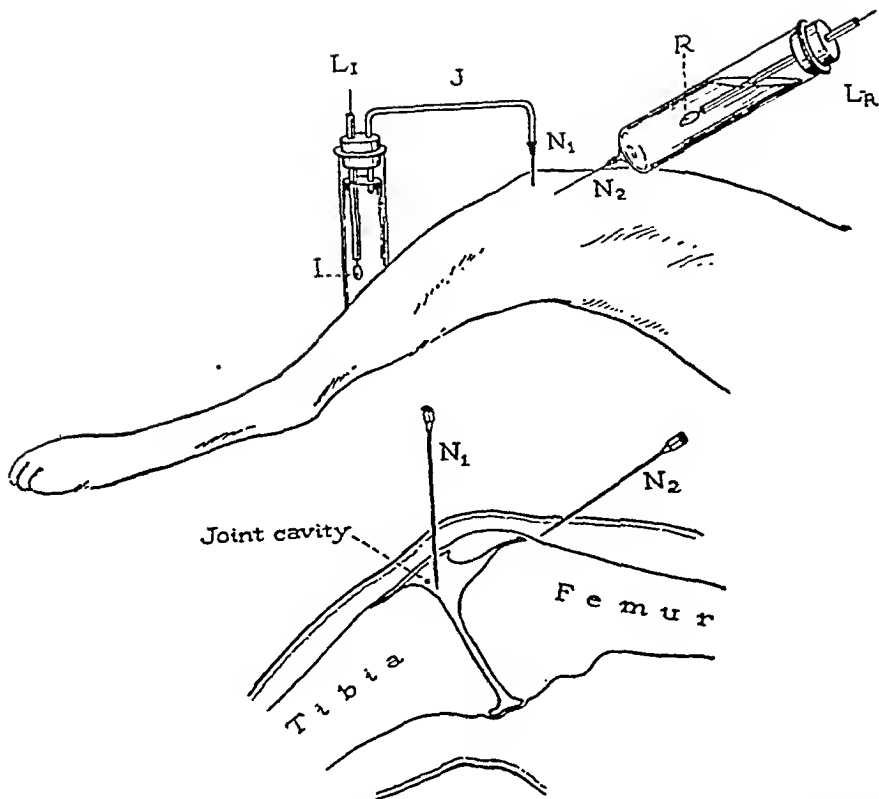


Fig. 1. METHOD OF INSERTION of needles and arrangement of electrodes. N_1 , joint needle; N_2 , subcutaneous needle. I , indicator Ag-AgCl electrode; R , reference Ag-AgCl electrode, each in 0.15 M NaCl. J , saturated KCl-agar bridge. L_1 and L_2 , leads to potentiometer.

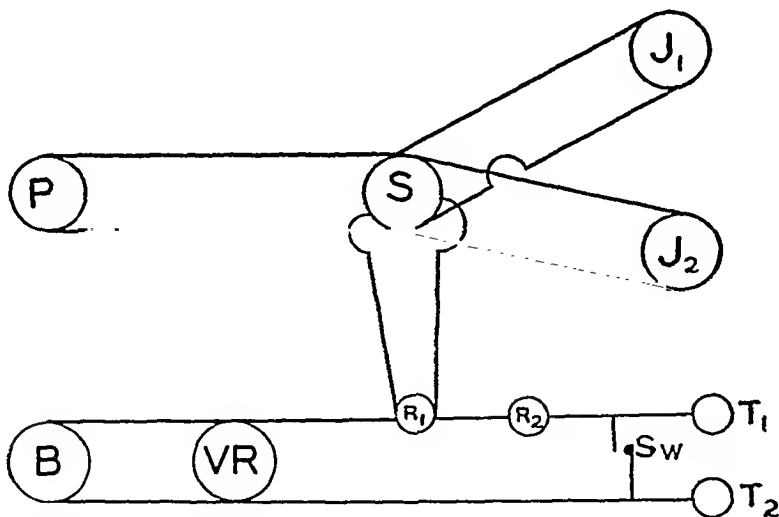
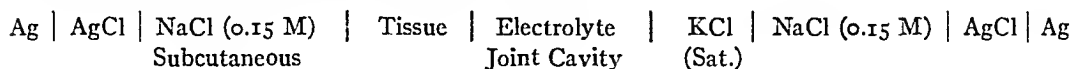


Fig. 2. DIAGRAM illustrating the circuit. P is the potentiometer, S is a selector switch used to connect right or left joint electrodes to P . S is also used to connect P to the voltage drop R , 5000 ohms in the polarization-depolarization circuit. R_1 is in series with R_2 , a 200,000 ohm fixed resistance. Current is supplied by a B battery tapped at 22.5 volts. Voltage is regulated by two variable resistances in series, VR . It is calibrated by closing a switch S_w and reading the potential drop across R_1 on P . T_1 and T_2 are terminals for the polarization and depolarization leads which are attached to either pair of joint electrodes. Resting depolarization curves are read with the voltage regulator set at zero volts. Polarization currents are determined by reading E_i , the potential drop across R_1 .

K-2 Leeds Northrup potentiometer and a galvanometer sensitive to 0.1 millivolt with this circuit. The schematic diagram of the experimental circuit is



Assuming the potential difference across the KCl bridge to be negligible, the observed E.M.F. represents the potential difference across the tissues.

In this arrangement, only the electrolyte within the joint cavity was varied by means of a 22-gauge needle inserted into needle N_1 . The fluid in N_1 was exchanged 3 or 4 times before reading. Accordingly, the experimental potentials observed represented variations at the inner surface of the cavity.

In the later phases of the investigation it was found desirable to study not only the potentials, but also currents across the membrane. These currents were produced in two ways. A depolarizing current was obtained by allowing the membrane to discharge through a known external resistance, and a polarizing current by applying a known external E.M.F. through the resistance. In each case the current was measured by determining with the potentiometer the voltage drop across a known part of the external resistance. Time variations of current were determined. In addition, it seemed desirable to determine the time variation of the membrane potentials after the polarization or depolarization of the membrane by these methods. To carry out these operations most conveniently, a control box was devised which facilitated these procedures. An outline of the circuit is given in figure 2, with a brief description of the operation.

EXPERIMENTAL

A. Resting membrane potentials. In determining resting potentials, dogs anaesthetized with nembutal were used. At the beginning of each experiment, electrodes were mounted in right and left legs as previously described. After refilling the needle N_1 three or four times with 0.15 M NaCl, the resting potential was measured. Solutions were removed and the cavity and needle were refilled with experimental solution until three consecutive readings agreed within 1 millivolt. In general, there was little difficulty in obtaining steady reproducible readings after the third or fourth. Duplicate series were generally run on both joints. Readings were made rapidly, usually at the rate of two per minute. The following anions were studied: chloride, bromide, iodide, sulfate, salicylate and benzoate. In several experiments both acetyl salicylate and acetyl-5-brom-salicylate ions were studied, each as the neutral sodium salt. The cations observed were the following: sodium, lithium, potassium, calcium and magnesium. When solubility permitted two concentrations of an electrolyte were studied; 0.15 molal for the uni-univalent type of salt, and 0.075 molal for the uni-bivalent type such as Na_2SO_4 or CaCl_2 . One-tenth dilutions of these solutions were also studied. The electrolyte at low concentration was rendered isotonic by making the dilution with isotonic (0.3 molal) glucose.

In studying any series of anions or cations, it was desirable to restandardize frequently the potentials of the isotonic NaCl solution. The resting potential of NaCl was taken for convenience as the basis of reference for comparison of other electro-

lytes. It was generally found to be negative, but usually less than ten millivolts. The sign, negative or positive, refers always to the sign of the indicator electrode.

A typical short series of determinations of resting potentials is given in table 1. The results of a large number of experiments were averaged in table 2, where the

TABLE 1. EXPERIMENT 21
Results on Resting Potentials. Details of Procedure

RIGHT LEG					LEFT LEG			
Time	Salt	Equiv. Conc.	Solution No.	Potential	Salt	Equiv. Conc.	Solution No.	Potential
<i>min.:sec.</i>				<i>millivolts</i>				<i>millivolts</i>
0:00	NaCl	0.15	1	-6.1	NaCl	0.15		
0:30							1	-3.1
0:45			1	-5.9				
1:15			2	-5.8				
1:45							1	-3.6
2:00			2	-5.8				
2:30							2	-3.4
3:00			3	-5.5				
4:00							2	-3.3
4:30			3	-5.5				
5:00	NaCl	0.015			KCl	0.15	3	-3.0
5:30			1	-18.5				
6:00			1	-17.4				
6:15							1	-7.2
6:45			2	-19.6				
7:30							2	-7.5
8:00			3	-18.9				
9:00							3	-7.6

TABLE 2. AVERAGE RESTING POTENTIAL OF VARIOUS STRONG ELECTROLYTES

SALT	EQUIV. CONC.	$E_{av} - E_{NaCl}$	STD. DEV.	NO. EXPERIMENTS
KCl	0.15	-3.4	± 2.0	11
LiCl	0.15	+3.3	± 2.0	11
CaCl ₂	0.15	+5.4	± 2.4	14
MgCl ₂	0.15	+8.0	± 3.1	15
NaBr	0.15	+5.1	± 2.7	20
NaI	0.15	+26.0	± 8.8	25
Na ₂ SO ₄	0.15	-7.9	± 2.5	6
Na Salicylate	0.15	-4.1	± 2.4	27
Na Benzoate	0.15	-6.5	± 2.7	6
NaCl	0.015	-17.0	± 4.4	30

values refer to the resting potential of NaCl. The results are arranged according to the Hofmeister series. Practically without exception the results on each animal gave the same series as that obtained from the average of all experiments. In addition, the potentials of 0.015 M NaCl are included, referred to 0.15 M NaCl as standard.

B. Depolarizing currents. In measuring depolarization of the articular structure, the control circuit of figure 2 was utilized. The resting potential was read on the open circuit. At a given time signal the circuit was closed through the 205,000-ohm combined resistance. The voltage drop, E_i , across 5000 ohms was read immediately on the potentiometer. The reading of the instrument was continued at 10- or 15-second intervals for one or two minutes. The circuit was reopened and the potential was again read as in the determination of the resting potentials. Currents were calculated by dividing E_i by 5000 ohms. The membrane potential at any instant was estimated by multiplying E_i by the factor 41, the ratio of 205,000 to 5,000.

A few typical observations and calculations are given in table 3 for various electrolyte solutions. A more extensive summary of the data is presented in table 4.

TABLE 3. EXPERIMENT 33
Resting Currents and Potentials. Period of Depolarization 1 minute

SALT	EQUIV. CONC.	RESTING POTENTIAL ¹ E	READING ON CLOSED CIRCUIT ¹ E_i	CURRENT I $E_i \times \frac{1}{5000}$	TOTAL RESISTANCE $R_t = \frac{E}{I}$	TISSUE RESISTANCE $R_x = R_t - 205,000$
		<i>millivolts</i>		<i>amps $\times 10^8$</i>	<i>ohms</i>	<i>ohms</i>
Na Salic.	0.15	-10.4	-0.169	-3.4	309,000	104,000
Na Benz.	0.15	-12.3	-0.180	-4.0	308,000	103,000
KCl	0.15	-3.0	-0.043	-0.9	333,000	128,000
CaCl ₂	0.15	+2.9	+0.042	+0.8	362,000	157,000
MgCl ₂	0.15	+6.0	+0.105	+2.1	286,000	81,000
MgCl ₂	0.015	-11.1	-0.181	-3.6	308,000	103,000
KCl	0.015	-10.0	-0.103	-2.1	476,000	271,000
NaCl	0.015	-6.4	-0.072	-1.4	457,000	252,000
LiCl	0.015	-7.5	-0.102	-2.0	375,000	170,000
CaCl ₂	0.015	-5.7	-0.068	-1.4	407,000	202,000

¹ In the 0.15 equivalent series, E_i and E were nearly constant over the period of depolarization, 1 minute. The readings given are initial values, the value of E_i being obtained about 10 sec. after closing the circuit. In the 0.015 equivalent series, E_i always fell rapidly at first, becoming steady after 15 or 20 seconds. The final constant currents are given. E is the membrane potential immediately after opening the circuit.

The calculations are based on Ohm's law and yield values for R_x , the resistance across the tissues. This is of the order of 100,000 ohms for the more concentrated solutions (0.15 equiv.), and much higher for the dilute solutions (0.015 equiv.). Accordingly, the depolarization currents can be approximately predicted from the resting potential by dividing the total of the circuit ($R_x + 205,000$). The variability of R_x with concentration must not be disregarded for large concentration changes.

It is also observed that the currents and potentials were much more stable in the 0.15 equivalent solutions than in the one-tenth dilutions. With the latter, the current fell to a rather small fraction of its initial value and then became steady. This usually occurred within 10 or 15 seconds. When the circuit was reopened, the potential was also found to have fallen in proportion, but rapidly returned to the

polarized initial value. Neither potential nor current fell appreciably when the electrolyte concentration was 0.15 equivalent.

In the dilute series of readings of polarization current given in table 3, final steady values of E_i are given and the potential is that at the end of the depolarization period.

C. *Polarization currents.* The circuit of figure 2 was employed in the following manner. The variable resistance was adjusted to control the output voltage. This

TABLE 4. MEMBRANE RESISTANCE CALCULATED FROM DEPOLARIZATION CURRENTS

SALT	EQUIV. CONC.	NO. OF EXPERIMENTS	R_x
			Mean and Std. Dev.
KCl	.15	5	97,000 \pm 12,000
	.015	4	212,000 \pm 33,000
NaCl	.15	7	85,000 \pm 10,000
	.015	7	233,000 \pm 41,000
LiCl	.15	5	101,000 \pm 15,000
	.015	4	251,000 \pm 31,000
CaCl ₂	.15	6	110,000 \pm 20,000
	.015	4	243,000 \pm 44,000
MgCl ₂	.15	3	96,000 \pm 11,000
	.015	3	220,000 \pm 28,000
NaBr	.15	4	91,000 \pm 15,000
	.015	4	218,000 \pm 36,000
NaI	.15	7	84,000 \pm 17,000
	.015	5	222,000 \pm 41,000
Na ₂ SO ₄	.15	4	107,000 \pm 16,000
	.015	2	251,000 +
Na Salicylate	.15	5	112,000 \pm 12,000
	.015	3	240,000 \pm 21,000
Na Benzoate	.15	3	105,000 \pm 17,000

was calibrated on the potentiometer, the leads of which were connected across the 500-ohm resistance. The sign of the voltage was controlled by a pole reverser in the power circuit, so that current could be sent in either direction. The sign of the current indicates the polarity connected to the indicator electrode of the joint. After calibration of the external voltage the selector switch was connected to the open circuit position for the desired joint, the calibration switch was turned off and the resting potential was read. At a given time signal the circuit was closed and readings of the potentiometer were made at frequent intervals, accurately timed with a stop

watch. The current was applied for a definite period, usually one or two minutes accurately timed.

Currents were calculated from the data by application of Ohm's law. The observed voltage drop across the 5000-ohm resistance when divided by 5000 yields amperage. When the applied voltage is divided by amperage, the total resistance R_t of the circuit is obtained. This represents the sum ($R_t + 205,000$) where R_t is the resistance across the tissues. Actually R_x is the total resistance between the leads of the silver chloride electrodes. The resistance through the reference electrode vessels and the KCl bridge is of a much smaller order of magnitude than R_x , which can be taken to represent the membrane resistance, with an error of less than 1000 ohms.

Detailed results are presented in table 5 for three experiments. The table also includes a summary of results of a much larger number of experiments. The results of the three detailed experiments are shown in figure 3. These indicate the relations between observed current and total voltage drop through the circuit. In addition, they illustrate the relation between current and the voltage drop E_x across the articular tissues. Details of the calculation are given in the tables. It is evident from the calculations that more constant values for R_t and R_x are obtained when the applied voltage was corrected by adding algebraically the membrane potential. The latter may either reinforce or oppose the applied voltage. The correction is relatively unimportant at high applied voltages, but is quite significant at 0.5 volt or less. It is clear from figure 3 that the relation between current and voltage is linear for low voltages but that the ratio of the two is greater for the 0.15 molal solution than for the dilute solution. This corresponds to the previously mentioned variation of R_x with concentration. Multiplying the current by R_x , the voltage drop across the tissues, E_x , is obtained.

It is also evident from figure 3 that the straight line relation between current and voltage holds accurately only in the low voltage range (± 0.5 volt). At higher voltages the curves are sigmoid and symmetrical about the origin. This is especially clear when current is plotted against E_x , the potential drop across the tissues. The deviation is in the direction of lower tissue resistance, R_x , at higher voltages. The deviation becomes quite pronounced when E_x is 1 volt or higher. The tendency for R_x to decrease is also evident from the calculations of table 5.

D. *Discharge potentials after polarization.* After running the polarization current for an accurately timed period, usually one or two minutes, the circuit was opened and the membrane potentials were read as in the case of resting potentials. It was found that the initial potential depends on the sign and magnitude of the applied voltage, the time of polarization and on the nature of the solution. As one would expect, the polarity of the membrane corresponds to that of the current source applied to the indicator electrode. When the polarity is positive the induced polarization is also positive. That is to say, current flows in the reverse direction when the external source is removed. The polarization potential varies with time, asymptotically approaching the resting equilibrium level. Two types of effects are distinguishable. At low voltages or for short periods of polarization, the potentials fell rapidly. Very often this decrease was seen to be linear when it was plotted ex-

ponentially against the time of discharge. This is clearly revealed by using semi-logarithmic graph paper with the displaced potentials plotted as ordinates and time as abscissa. Time of discontinuing the polarization current is taken as zero. Ordinates are plotted as $\pm \Delta E_p$, the difference of observed polarization potential and

TABLE 5. VARIATION OF CURRENT WITH APPLIED VOLTAGE

APPLIED VOLTAGE	CORRECTED FOR MEMBRANE POTENTIAL	E_1 VOLTS $\times 10^1$	CURRENT 1 $\frac{E_1}{5000} \times 10^3$	R_x (OHMS)		E_x (VOLT) IR_x CORR.
				Uncorr.	Corr.	
<i>Experiment 34—0.15 M NaCl</i>						
0.00	+0.011	+1.9	+3.8	—	71000	+0.003
-0.050	-0.039	-6.4	-12.8	186000	100000	-0.013
+0.050	+0.063	+10.8	+21.6	27000	87000	+0.019
-0.100	-0.087	-15.0	-30.0	128000	85000	-0.025
+0.100	+0.114	+19.8	+39.6	47000	83000	+0.033
-0.250	-0.237	-41.9	-83.8	95000	80000	-0.067
+0.250	+0.265	+46.2	+92.4	65000	82000	+0.076
-0.500	-0.490	-87.6	-175.2	80000	75000	-0.131
+0.500	+0.514	+90.1	+180.2	73000	73000	+0.155
-1.00	-1.00	-180	-360	73000	73000	-0.262
+1.00	+1.01	+182	+364	60000	61000	+0.266
-2.00	-2.00	-379	-758	64000	64000	-0.486
+2.00	+2.01	+365	+730	69000	69000	+0.518
-3.00	-3.00	-555	-1110	65000	65000	-0.721
+3.00	+3.01	+565	+1130	60000	61000	+0.690
-4.00	-4.00	-735	-1470	67000	67000	-0.985
+4.00	+4.01	+740	+1480	65000	66000	+0.975
-10.00	-10.00	-2008	-4016	44000	44000	-1.77
+10.00	+10.00	+1992	+3984	46000	46000	+1.83
<i>Experiment 35—0.15 M NaCl</i>						
-1.00		-162	-324	104000		-0.337
+1.00		+170	+340	89000		+0.302
-2.00		-339	-678	90000		-0.610
+2.00		+348	+696	83000		+0.577
-4.00		-709	-1418	78000		-1.12
+4.00		+716	+1432	75000		+1.07
-6.00		-1087	-2174	72000		-1.56
+6.00		+1098	+2196	69000		+1.52
-10.00		-1949	-3898	52000		-2.02
+10.00		+1960	+3920	51000		+2.00

equilibrium (resting) potential. The sign applied is that of the potential. Some linear polarization potential curves are shown in figure 4.

The second type of effect is that produced by high voltage, 4 volts or more, applied for two or more minutes. When this effect is plotted on logarithmic paper, it is found that there was a rapid initial fall of potential which reached a steady level of about 150 millivolts from the equilibrium level. This level may be maintained

for a period up to seven or eight minutes, producing a plateau in the curve. Eventually there was a rapid fall of potential to the equilibrium level. At intermediate

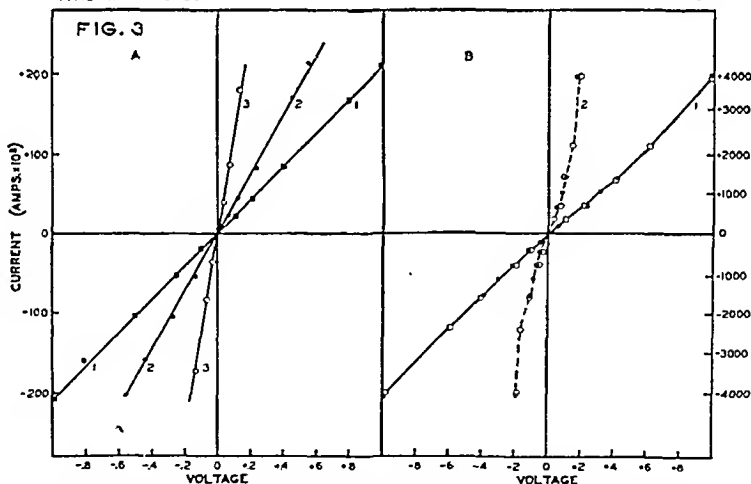
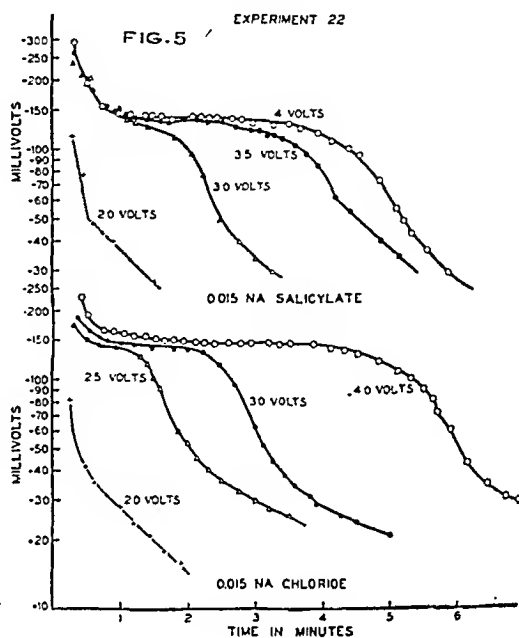
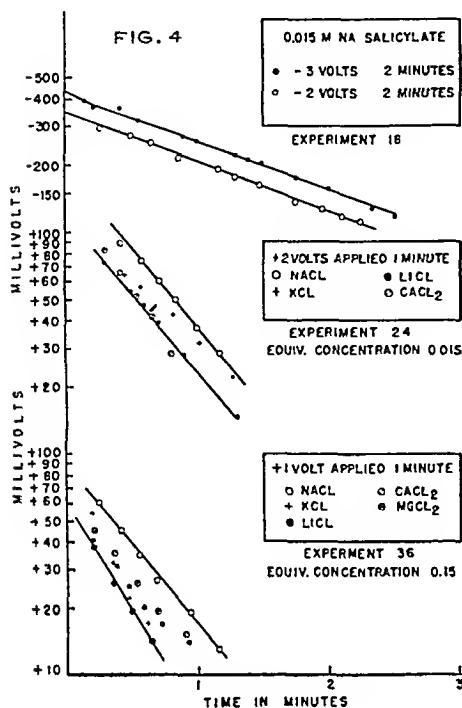


Fig. 3. A. CURVE 1, EXPT. 42. Current plotted against total applied voltage. Curve 2, expt. 42. Current plotted against voltage drop across tissues. Electrolyte, 0.015 M NaCl + 0.270 M glucose. Curve 3, expt. 34. Electrolyte, 0.15 M NaCl. Current plotted against tissue voltage drop. B. Curve 1, expts. 34, 35; closed dots, 34, open dots, 35. Current plotted against total applied voltage. Curve 2, expts. 34, 35. Current plotted against voltage drop through tissues.

Fig. 4. LINEAR DEPOLARIZATION POTENTIAL CURVES. Ordinates, ΔE_p plotted on logarithmic scale. Abscissa, time in minutes.

Fig. 5. DEPOLARIZATION CURVES at higher voltages. Time of charging two minutes. Upper curves, Na salicylate; lower curves, NaCl ΔE_p plotted on logarithmic scale.

voltages the plateau shortened, the type of curve undergoing a transition to the linear type of depolarization. It is characteristic that the plateau, when present, occurred

at a nearly constant level, generally displaced 150 millivolts or 200 millivolts from the equilibrium level. It was maintained longer when dilute electrolyte was transferred than in the case of the 0.15 M solutions. It thus, evidently, depends on R_x , the tissue resistance. Accordingly, low values of R_x appear to facilitate rapid depolarization of the membrane. The nature of the effects with dilute sodium salicylate and sodium chloride are evident from figure 5. In both cases the applied voltage was negative to the joint. Very similar effects are obtained, however, by reversing the current. They were obtained with ions of either sign, monovalent or divalent. In the discharge curves after application of 2 volts, the linear relation is seen to hold after about one-half minute. The rate of discharge at every voltage was approximately the same after the period of rapid discharge began.

RESULTS

A. *Relation of resting potentials to ionic mobilities.* As a basis for quantitative calculations, it will be assumed that the resting potentials obtained with simple inorganic ions are essentially diffusion potentials or liquid junction potentials. Biological effects such as secretory or metabolic processes are probably involved in the behavior of the tissues with respect to many substances. With certain inorganic and organic ions, however, the laws of electrolytic diffusion will be applied as the basis of theoretical treatment.

The theory of Henderson (2) applied to liquid junction potentials leads to the relation

$$E_d = (t_1 - t_2) \frac{RT}{F} \ln \frac{C_1}{C_2}. \quad (1)$$

E_d is the diffusion potential at a liquid junction; t_1 is the Hittorf transference number of the cation; t_2 is that of the anion; and C_1 and C_2 denote the concentrations of the two solutions. R , T and F denote, respectively, the gas constant, the absolute temperature and the Faraday constant.

The equation is applied as follows: From the data of table 2, the average value of the membrane potential of 0.015M NaCl is 10.7 millivolts negative to 0.15 M NaCl. Substituting this value in equation 1 and converting the constants, it is found that $(t_1 - t_2) = -\frac{17.0}{61.6}$, where the temperature is taken as 37°C.

Since the sum of the transference numbers is unity, $t_1 = 0.36$ and $t_2 = 0.64$. In water, from conductance data, $t_1^\circ = 0.408$ and $t_2^\circ = 0.592$ at infinite dilution.² These values lead to a theoretical diffusion potential of -11.4 mv. The difference is within the standard deviation of the measurements in table 2.

The type of determination in which the potential is measured between equally concentrated solutions of salts having one ion in common corresponds to that made by Lewis and Sargent (6).

In the case of uni-univalent salts the applicable formulae are those of Planck (9) where

$$E_d = \frac{+RT}{F} \ln \frac{(U_{Na} + U_{x-})}{(U_{Na} + U_{Cl})} \quad (2)$$

and

$$E_d = -\frac{RT}{F} \ln \frac{(U_{x+} + U_{Cl})}{(U_{Na} + U_{Cl})}. \quad (3)$$

Here U_{x-} and U_{x+} denote the ionic mobilities of the anion or cation that is substituted for chloride

² Data obtained from International Critical Tables.

or sodium in the joint, while U_{Na} and U_{Cl} denote the mobilities of these ions in the tissues. For interpreting the results, it is convenient to refer all mobilities to sodium, since the concentration of sodium ions in the body fluids approximates 0.15 M. It is then found that

$$E_d = + \frac{RT}{F} \ln \frac{\left(1 + \frac{U_{x^-}}{U_{Na}}\right)}{\left(1 + \frac{U_{Cl}}{U_{Na}}\right)} \quad (4)$$

and

$$E_d = - \frac{RT}{F} \ln \frac{\left(\frac{U_{x^+}}{U_{Na}} + \frac{U_{Cl}}{U_{Na}}\right)}{1 + \frac{U_{Cl}}{U_{Na}}} \quad (5)$$

Accordingly, from the observed average values of the membrane potentials, the apparent relative ionic mobilities of anions or cations can be estimated, referred to sodium ions. Even in aqueous systems these equations are to be considered as approximation formulae. The ratios will be interpreted as indicating only apparent mobilities. In applying formulae (4) and (5) the value 1.78 is taken for the ratio $\frac{U_{Cl}}{U_{Na}}$. This is obtained from the dilution data on NaCl and represents the ratio $\frac{t_2}{t_1}$.

The results of the calculation are presented in table 5. Apparent relative mobilities in the tissue are compared with relative mobilities of the same ions in water at 37°C. The results indicate that the relative mobilities of Li^+ and K^+ are about the same in the tissues as in water. Values for Cl, Br and I are all increased, I most markedly. The bivalent cations, Ca^{++} and Mg^{++} , show much lower relative mobilities in the tissue than in water. Salicylate and benzoate, like the other monovalent anions, show greater relative mobilities in the tissue. Their mobilities are, however, lower than that of the chloride ion.

The results can be arranged according to the Hofmeister series in increasing order of mobilities.

Tissue

Anions: Benzoate < Salicylate < SO_4^- < Cl^- < Br^- < I^-

Water

Benzoate < Salicylate < Cl^- , Br^- , I^- < SO_4

Tissue

Cations: Mg^{++} < Ca^{++} < Li^+ < Na^+ < K^+

Water

Li^+ < Na^+ < Mg^{++} < Ca^{++} < K^+

The most conspicuous effects are the displacement of Ca^{++} and Mg^{++} to the left of the series (low mobility in tissues) and the very pronounced shift of I^- to the right. The tissues apparently absorb iodide ions easily and bivalent cations poorly. The

relatively low mobilities of salicylate and benzoate are probably related to their large molecular volumes compared to the other ions. Yet their apparent mobility relative to sodium is about twice as great in the tissues as in water. Evidently other physicochemical factors such as lipoid solubility or adsorption are involved.

The results on the elementary ions are presented graphically in figure 6. Apparent relative mobilities are plotted against atomic numbers. The graph also includes the relative mobilities in water. Several of the ions have approximately the same apparent relative mobilities in tissues as in water. The most striking exception shown on the diagram is iodide ion.

B. Ions with unstable potentials. Several other ions besides those recorded in table 5 have been studied. Certain of these gave as a rule unstable potentials, and for that reason have not been included. Thiocyanate ion nearly always showed a high initial positive potential, rapidly drifting negative. Iodide ion occasionally showed a similar effect. In those cases where the potential drifted, a value was not included in calculating the averages.

In addition, two salicylate derivatives were studied—the sodium salts of acetyl salicylate and of acetyl-5-brom-salicylate. The former was studied at 0.015 equivalent and the latter at 0.010 equivalent concentrations. The solubilities of the salts did not permit determinations at higher concentrations. Each of these salicylate derivatives gave in many cases potentials more negative than that of NaCl or Na salicylate at the same concentration. However, in a number of other cases each had the thiocyanate type of behavior, showing a high positive initial potential with a steady negative drift. The effect resembles that described by Höber with thiocyanate on the resting potential of frog muscle (3, 4). Because of the nature of these effects, it is evident that the diffusion potentials involve other factors than those determining the potentials given in tables 2 and 5. The nature of these factors will require further elucidation. Usually the effects are quite reversible.

C. Ionic antagonisms. Unstable or drifting potentials were also often encountered with mixtures of two different cations. These were especially noted with mixtures of a univalent and a bivalent cation. When the effect occurred, it was observed typically that the potential of the mixture was more negative than that of either of the pure component ions. Thus, a one-half dilution of 0.15 M NaCl with 0.075 M CaCl_2 would sometimes show a potential considerably more negative than that of either of the pure salts. The effects were not sufficiently reproducible to justify taking quantitative averages. However, there were some individual experiments that yielded results that are of interest. One such experiment is presented graphically in figure 7.

Plotted as the abscissa are the equivalent ratios of Na and Ca, or of Na and Mg in a binary mixture. These ratios represent equivalents of one ion divided by the total equivalents of the solution. The pure salts are represented by the ratio 1.0. In this experiment, the starting point was at *A* in the 0.075 M CaCl_2 solution where the observed potential was about -6 millivolts (upper half of figure 6). This solution was replaced by mixtures in which part of the Na was replaced by Ca, at points *B*, *C* and *D*. There was little change of potential. *E* represents pure 0.075 M CaCl_2 and *F* a one-tenth dilution of this, which caused the potential to go approximately

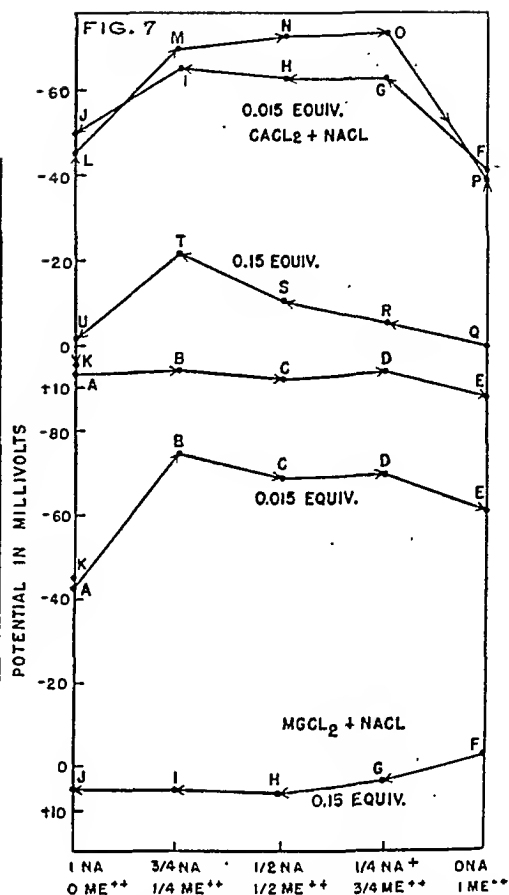
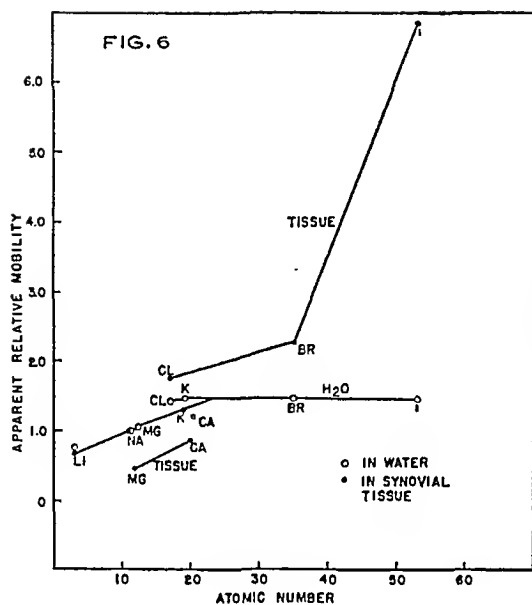


Fig. 6. APPARENT RELATIVE MOBILITIES of inorganic anions and cations plotted against atomic number. Values for Na taken as unity in water and in synovial tissue.

Fig. 7. ANTAGONISTIC ION EFFECTS in salt mixtures. Upper curves, $\text{CaCl}_2\text{-NaCl}$; lower curves, MgCl_2 . Ordinates, potential in millivolts. Abscissa, fraction of total equivalent concentration due to each ion.

TABLE 6. APPARENT RELATIVE MOBILITIES OF THE IONS

ION	ATOMIC NUMBER	$\frac{U_{x\pm}}{U_{\text{Na}^+}} \text{ (TISSUES)}^1$	$\frac{U_{x\pm}^0}{U_{\text{Na}^+}^0} \text{ (WATER)}^2$	$(E - E_{\text{NaCl}}) \text{ (AVE.)}$
				millivolts
Li^+	3	0.68	0.77	+3.3
Na^+	11	1.00	1.00	0.0
K^+	19	1.34	1.49	-3.4
Mg^{++}	12	0.49	1.06	+8.0
Ca^{++}	20	0.90	1.19	+5.4
Cl^-	17	1.78	1.44	0.0
Br^-	35	2.36	1.48	+5.1
I^-	53	6.87	1.46	+26.0
SO_4^{--}		1.76	1.50	-7.9
Salicylate		1.38	0.60	-4.1
Benzoate		1.18	0.56	-6.5

¹ Calculated from formulae of Planck (9) and Henderson (2).

² Aqueous mobilities estimated at 37°C. from conductance data in International Critical Tables.

50 millivolts negative. The cycle was completed in the counter-clockwise direction through the points *G*, *H*, *I*, *J* and *K*, where the potential was about -4 millivolts. Then the cycle was reversed by varying the solutions in the order *L*, *M*, *N*, *O*, *P*, *Q*, etc. and was completed again at *U*. The ionic antagonisms are clearly shown in the sections *F-L*, *J-P* and *Q-U*, where the potentials of the mixtures are more negative than those of the pure salts.

In the lower part of figure 7, the experiment is repeated with *Mg* in place of *Ca*. A clockwise cycle begins at *A* and ends at *K*, following the arrows. The antagonistic effect is noted in the dilute series (0.015 equiv.) but is not observed in the concentrated range.

It should be pointed out that with each of the three salts, the dilution effect found with this animal was much larger than that usually encountered. The average effect with *NaCl* is about -17 millivolts with a one-tenth dilution. In this case it was about -50 millivolts on each of the salts, and it was quite reproducible through three cycles, each of which included three dilutions. On the basis of the theory of diffusion potentials, a potential of -60 millivolts for tenfold dilution corresponds to complete cation impermeability. Dilution potentials of this order of magnitude were found in about ten per cent of the experiments.

D. Ringer's solution. In a number of experiments, observations were made on resting potentials and currents with mammalian Ringer's solution. The values were always very close to those for 0.15 *M NaCl* solutions, usually within ± 2 millivolts. The algebraic average of these positive and negative differences from the resting potential of *NaCl* was -0.5 mv. in seven experiments. This is not a sufficient difference to be considered significant.

DISCUSSION

An exact theory of liquid junction potentials depends on a knowledge of ionic activities, transference numbers and the precise conditions of mixing at the liquid boundary. Even in simple liquid junctions, the exact mathematical solution of the problem depends on the evaluation of integrals involving variations of transference numbers and ionic activities across the boundary between two liquids (10). This evaluation becomes especially difficult where more than one electrolyte is involved, when there is more than one concentration gradient and when ions of different valences are to be considered. All of these difficulties are met in problems involving diffusion in physiological systems. Accordingly, the calculation of apparent ionic mobilities, according to the equations of Planck and Henderson, is of the nature of a semiempirical approximation.

As such, the calculation indicates that for numerous ions in the synovial tissues the apparent relative mobility, referred to sodium ion, is close to that in water. For these ions, therefore, the permeability of the membrane depends primarily on the aqueous mobility of the ion. The alkali ions, chloride and sulfate ions belong in this category. Other ions appear to have greater relative mobilities in the tissues than in water. Iodide, bromide, salicylate and benzoate belong to this group. On the other hand, bivalent cations have lower apparent mobilities in tissue than in water. These results appear to indicate that hydration of the ions, or the position of the ions in the

lyophilic series, as well as the mobility in water determine the diffusion in synovial tissues. High molecular volume in the case of substances such as benzoate and salicylate would be a factor lowering mobility in both water and tissues.

It seems noteworthy that in many cases the diffusion potential across a physiological membrane of high resistance (100,000 ohms) should correspond closely to that estimated from the Planck and Henderson formulae. This indicates that, except for iodide and a few other ions studied, there is little selectivity of the membrane at the inner surface of the joint cavity. Accordingly, one would picture a porous type of membrane, with a high internal resistance, permitting diffusion of most ions in proportion to their aqueous mobilities but of much less magnitude.

It has been shown that the membrane resistance decreases rapidly when the voltage drop across the membrane is greater than 1 volt. The current in that case is about 10 microamperes or greater. As the current is increased in either direction, resistance decreases, as shown in figure 4. It is possible that this indicates an increased electro-osmotic flow of water with the current. If this were the case, then a flow of water produced by a pressure gradient across the membrane would be expected to bring about a change of the membrane potential. The mechanism corresponds to the streaming potential of colloidal electrochemistry.

The mechanisms involved in the variations of potential and current during polarization and depolarization may also be discussed from the point of view of physicochemical liquid junction potentials. Logarithmic depolarization curves of the type shown in figure 4 represent the type of curve obtained with discharge of an electrolytic condenser through a constant resistance. Current at any instant is proportional to difference of potential across the conductor. Difference of potential is proportional to the charge or to the polarization potential. The integrated form of the equation for E is $\ln E = \ln E^\circ - \frac{t}{RC}$ where E° is the polarization potential at zero time, R denotes resistance in ohms, C capacity in farads and t is the time in seconds. From figure 4 the value of C can be estimated. The time for the potential to fall to half its value at any instant is about 30 seconds for the two lower sets of curves and about 60 seconds for the upper curves. Substituting 100,000 ohms as the value of R , C is seen to be of the order of 100 microfarads.

In figure 5 it is shown that the membrane can maintain a nearly constant potential difference of about 150 millivolts for several minutes. During this time, a current of approximately 0.75 microampere is produced, if R is estimated as 200,000 ohms. Accordingly, about 45 microcoulombs of charge are transferred per minute or about 5×10^{-10} equivalents of ions. Taking the volume of the joint fluid as 1 cc., this is of the order of a concentration change of 5×10^{-10} equivalents per liter. These ions are evidently stored at constant potential difference. The mechanism of the depolarization process might be pictured as follows. During the flow of polarization current across the membrane, the polarization vector is along the lines of force normal to the membrane surface. When the current is cut off, depolarization immediately begins. The first step to be predicted is the equalization of the potential at the membrane surfaces, the ions flowing tangentially along the surfaces from the points of highest polarization. The membrane potential, which is measured in the

direction of the initial lines of force, falls rapidly until the potential of each surface is equalized tangentially. Simultaneously there is also depolarization normal to the surface produced by current across the membrane. When the membrane potential reaches a value of about 150 millivolts, it becomes constant. This represents a difference of potential extended across the membrane in the direction normal to both surfaces. When the excess of ions (5×10^{-10} equivalents per minute) has been discharged, the membrane begins to depolarize according to the logarithmic law. The value 150 millivolts thus represents a saturation value for the membrane. For a capacity of 100 microfarads the charge of the membrane would be 15 microcoulombs, or about one-third the quantity discharged per minute in the steady state at 150 millivolt potential difference.

In the light of these ideas, it is possible to explain the effects observed with resting depolarization currents. It was found that steady currents and potentials were obtained with all salts at 0.15 equivalent concentration. With the one-tenth dilutions, rapidly decreasing currents and potentials were observed, resulting in a final steady state. In the latter case, a final potential of -10 millivolts produced a current of about -2×10^{-8} amperes as a steady state. The initial potential and current were about twice these values and fell to the steady state in about 15 seconds. This corresponds to an initial charge of 2 microcoulombs, and a final charge of 1 microcoulomb. Taking an average current of -3×10^{-8} amperes for 15 seconds the transfer of charge is about 0.5 microcoulomb. In the steady state, when the current is -2×10^{-8} amperes, this represents a transference of electrolyte at the rate of 0.72×10^{-11} equivalents per minute, taking the transference number of the anion as 0.6. This would represent a change of concentration within the joint cavity of 0.72×10^{-8} equivalents per liter per minute, taking the volume of the joint fluid as 1 cc. In the steady state anions and cations are being transferred at equal rates. At this point the rate of depolarization, as measured by current through the outer circuit, equals the rate of polarization determined by the difference of mobility of the ions.

The steady resting currents obtained with salts at 0.15 equivalent concentration are also readily explained. In this case there is no concentration gradient for the ion that is common to both liquids. With the circuit closed, this ion carries current at approximately constant (zero) gradient. Of the other two ions, the more mobile one carries current across the membrane; the other is involved in the electrode reaction. Thus, with NaI in the joint, iodide crosses the membrane, carrying nearly 90 per cent of the current, while sodium migrates in the opposite direction carrying the remainder. Chloride deposited at the reference electrode and liberated at the indicator electrode is equivalent to the sum of iodide removed from the cavity and sodium entering it. With the currents and times involved, the equivalents transferred are of the order of 10^{-7} per liter. The membrane reaction is of the nature of an exchange of anions or cations depending on concentration gradients and individual ion mobilities. With 0.015 equivalent solutions in the joint cavity, the process at the membrane always involves flow of the ion of greater mobility in the direction of the concentration gradient. This determines direction of current flow in the outer circuit and the migration of the less mobile ions.

It is evident that from the theory of diffusion potentials, not only the magnitude and sign of the potentials and currents are predictable but also the stability under various conditions. Quantitative transference of various ions across the synovial membrane can, therefore, be estimated from electrochemical and bioelectrical data.

SUMMARY

1. Resting potentials across the synovial membranes of the knee joints of dogs have been determined *in vivo* for the alkali and alkaline earth cations and for halides, sulfate, benzoate, salicylate ions and some salicylate derivatives.

2. In a closed circuit across the membrane, currents have been determined for the same electrolytes with or without applied external voltage.

3. From the membrane potential and current values, resistance of the tissues in ohms has been estimated. For low applied voltages, current is directly proportional to E.M.F. corrected for membrane potential. At higher voltages ohmic resistance decreases.

4. Polarization potentials have been observed after applying current and the rates of depolarization determined.

5. By means of the Planck-Henderson theory of diffusion potentials, apparent relative mobilities of the ions have been calculated from the data. In many cases, relative mobilities have been found to correspond closely to the values calculated in aqueous solutions. Consistent Hofmeister series effects have been observed.

6. Antagonistic ion effects, the effects of concentration changes and effects of ions with unstable potentials are described.

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DISTRIBUTION OF POTASSIUM AND SODIUM BETWEEN SERUM AND CERTAIN EXTRACELLULAR FLUIDS IN MAN¹

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AN ESTIMATION of the concentrations of several substances in interstitial fluid surrounding the cell may be made under certain circumstances by determining their concentrations in serum. With respect to diffusible ions, the distribution across the 'vascular membrane' is believed to be in accord with the Gibbs-Donnan equilibrium. Many data have been reported which support the conclusion of Loeb, Atchley and Palmer (14) that "the relationships between serum and edema fluids result from a simple membrane equilibrium, influenced in part by the proteins present" (5, 6, 7, 9, 10, 15). Clearly, the equilibria attained by chloride, bicarbonate and sodium are in such good agreement with the theoretical ratios that this principle has been well established.

The equilibria reported for potassium (as well as for calcium and phosphate), however, indicate that a significant proportion of this ion in the serum might have been in a nondiffusible state (bound). Inspection of the reported data reveals an extraordinary degree of variation in the K equilibria which existed between serum and various extracellular fluids (pleural, peritoneal and subcutaneous); the ratios of concentration in fluid water to concentration in the serum water ($R_{sf}K$) varied from 0.40 to 1.07. If such unpredictable deviations from the anticipated theoretical equilibrium ratio for a freely diffusible ion do indeed exist, then any estimate of the [K] of the extracellular fluid from analysis of the serum would be liable to enormous error.

Investigations in this laboratory of the effect of [K] in extra- and intracellular *milieux* on neuromuscular function required a reliable means of estimating the [K] of interstitial fluid. We have, therefore, re-examined the distribution of K and of Na between the serum and certain readily obtained extracellular fluids in man.

METHODS

Sera and body fluids were analysed for K and Na in a modified Berry-Chappell-Barnes internal standard flame photometer (1). The photometer was designed as a null-point instrument. The sensitivity of the circuit was such that the precision vernier dial readings were reproducible to within 0.005 mEq/L. of K and 0.01 mEq/L. of Na. The internal standard, lithium, was added to both standard and unknown solutions to effect a final concentration of 100 p.p.m. for K analyses and 800 p.p.m.

Received for publication April 16, 1948.

¹ Work performed under contract between the Office of Naval Research, U. S. Navy Department, and The Johns Hopkins University.

for Na analyses. The overall accuracy of this instrument is indicated by the following studies.

Recovery of added K from 22 biological fluids (sera, exudates and transudates) and 12 solutions of sucrose varying in concentration from one to 20 per cent yielded a mean error of 1.0 per cent. The spread of errors is indicated in the following table.

Error, in %.....	0-0.9	1.0-1.9	2.0-2.9	3.0-3.9	4.0-4.9	7.0
No. of analyses.....	15	6	7	4	1	1
Cumulative %.....	44	62	82	94	97	100

Seventeen consecutive duplicate dilutions and analyses of biological fluids yielded a mean difference between the two samples of 0.1 mEq/l. The distribution of differences is presented below.

Difference in mEq./l.	0.0	0.1	0.2	0.3	0.4	0.5
No. of analyses.....	6	4	4	1	1	1
Cumulative %.....	35	59	82	88	94	100

Sera and fluids were diluted 1:10 for K and 1:100 for Na analyses. Volumetric errors⁴ introduced by viscosity (10) were minimized by measuring samples between marks. All analyses were performed in duplicate.

Samples of blood and fluid were drawn within the space of a few minutes. Whole blood was drawn into dry, oiled syringes from artery or vein without stasis and subjected to immediate centrifugation under oil. When in the course of a few minutes the cells had been partially thrown down, clear supernatant serum was pipetted off and recentrifuged at 3000 r.p.m. for 15 minutes. The upper two thirds of the separated serum was immediately pipetted off for analysis. The possibility of hemolysis was excluded by spectroscopic examination. The fluids similarly were centrifuged at high speed under oil. Subcutaneous edema fluid was collected through hypodermic needles or Southey tubes.

The concentration of protein in serum, pleural and peritoneal fluids was determined by a macro-Kjeldahl method (2). The concentration of protein in subcutaneous edema fluid was determined by a modified micro-Kjeldahl method (12). The nonprotein nitrogen concentration of subcutaneous fluid was assumed to equal that determined in the serum (4).

The proportion of water in the various fluids and serum was calculated as 100—the protein content in grams/100 ml.

The patients who were studied suffered from a variety of disorders in which there was accumulation of body fluids. None was significantly acidotic as judged by the carbon dioxide combining power of the serum.

RESULTS

The distribution of K and of Na between serum water and other extracellular water in the 22 cases studied is presented in table 1. The mean $R_{st}K$ was 0.92 (range: 0.82-1.03); the mean $R_{st}Na$ was 0.96 (range: 0.91-1.00). It will be noted from inspection of the data that no correlation exists between the value of the distribution ratio and the concentration of protein in either serum or extracellular fluid.

TABLE 1. DISTRIBUTION OF K, NA AND PROTEIN BETWEEN SERA AND CERTAIN EXTRACELLULAR FLUIDS

PATIENT	DIAG-NOSIS	SOURCE	K		Na		PROTEIN		RATIO $\frac{\text{FLUID}}{\text{SERUM}}$	
			fluid	serum	fluid	serum	fluid	serum	K	Na
			<i>mEq. per liter of water</i>				<i>grams %</i>			
Si	S	E V	4.4	5.2	147.5	150.5	0.6	4.8	0.85	0.98
We	C	E V	4.6	4.5	—	—	0.2	6.1	1.02	—
Fa	C	E V	4.6	5.2	144.5	150.5	0.3	6.4	0.89	0.96
Wa	C	E V	3.1	3.5	123.7	132.8	0.7	7.4	0.89	0.93
Wa	C	E V	6.4	6.7	126.2	128.2	2.0	6.8	0.96	0.98
Li	C	E V	4.4	4.5	138.3	147.2	0.7	6.0	0.98	0.94
Ma	C	E V	4.2	4.8	137.9	147.8	0.3	6.6	0.88	0.93
Fo	C	E V	5.7	6.6	128.2	130.2	0.1	6.1	0.86	0.98
Re	C	E A	4.1	4.7	133.0	142.8	0.4	5.0	0.87	0.93
Sm	C	C V	4.1	4.7	134.2	136.7	1.6	6.0	0.87	0.98
Sh	T	C V	4.4	4.4	—	—	5.7	7.1	1.00	—
Hi	C	C V	3.9	3.8	138.4	138.2	5.1	6.7	1.03	1.00
Hi	C	C V	3.9	4.5	122.2	134.7	4.0	7.2	0.87	0.91
Kr	C	C V	4.5	5.2	135.1	146.2	2.0	7.5	0.87	0.93
Lc	L	C V	4.1	4.3	145.4	147.0	0.9	5.6	0.95	0.99
Go	P	C V	5.1	5.4	144.8	145.8	5.7	6.5	0.94	0.99
Bu	C	C V	3.9	3.9	138.3	144.0	1.1	7.1	1.00	0.96
Ni	T	C V	4.5	5.5	138.0	137.8	4.0	6.0	0.82	1.00
Sa	L	P V	3.9	4.2	—	—	1.0	5.1	0.93	—
Br	L	P V	4.1	4.4	142.5	144.7	2.2	4.9	0.93	0.99
Lc	L	P V	4.9	5.6	134.8	138.7	0.9	6.6	0.88	0.97
Ta	L	P V	4.4	4.6	134.2	141.7	1.2	6.1	0.96	0.95
Mean.....									0.92	0.96
S. D.....									0.06	0.03
S. E. of mean.....									0.01	0.007

Diagnosis:

C—Cardiac failure
L—Cirrhosis
P—Pneumonia

S—Sprue
T—Tuberculosis

Source of Specimens:

E—Edema
C—Pleural fluid
P—Peritoneal fluid
V—Venous serum
A—Arterial serum

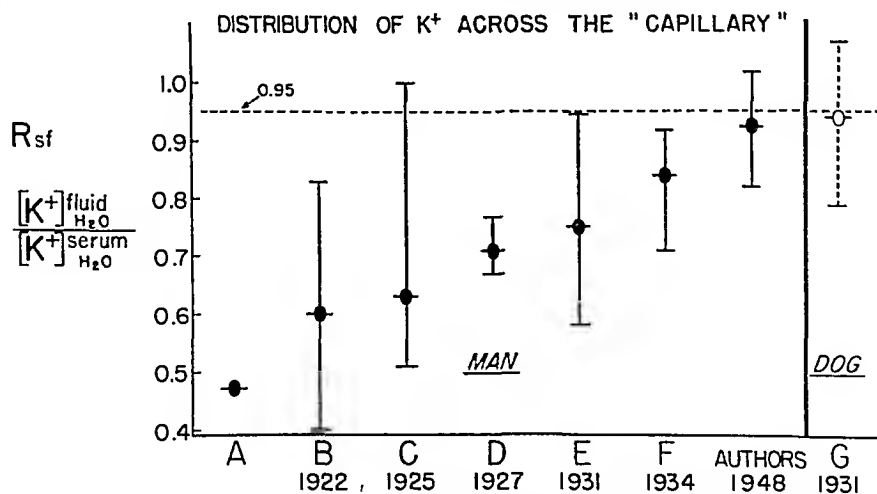


Fig. 1. MEAN DISTRIBUTION RATIOS are indicated by solid circles and the range of individual determinations by the vertical lines. The investigators are identified by the following references: A (15), B (14), C (6), D (9), E and G (7), and F (5).

DISCUSSION

The distribution ratios for K which have been reported previously for 50 individual determinations by six groups of investigators are summarised in figure 1. The mean $R_{sf}Na$ of these studies was 0.96 (range: 0.94–1.01). With the passage of years the mean $R_{sf}K$ has risen steadily toward that value found by most investigators to define the distribution of Na, Cl and HCO_3 . In the present study the difference between K and Na is small but statistically significant.

There are several possible explanations for the observed difference between $R_{sf}K$ and $R_{sf}Na$.

1. *Analytical factors.* Technics for estimating the concentration of K in biological fluids have undergone periodic improvements. Some early workers suspected that methodological difficulties accounted for the erratic and low $R_{sf}K$ which they observed (16). This factor may underlie certain wide variations in the reported data.

2. *Escape of K from erythrocytes.* Shed human blood undergoes a significant redistribution of K between cells and serum. When blood is held at 7°C. the escape of K from cells increases the serum concentration at a linear rate of 0.1 to 0.3 mEq/l./hr. At 25°C. K moves in the same direction, but at 37°C. erythrocytes remove K from serum (3, 8).

The usual technic for obtaining serum permits a period of at least 15 to 20 minutes to elapse between drawing the whole blood and separating the serum. This provides time for the transfer of sufficient K from cells to serum to increase the serum concentration by approximately 0.03 to 0.1 mEq/l. Furthermore, when serum is separated from clotted blood and then subjected to high speed centrifugation, a thin layer of red blood cells frequently may be seen lining the bottom of the vessel. Thus, the usual technics which are employed provide an ample opportunity for the escape of small but significant amounts of K from cells to serum. Theoretical calculations indicate that an escape of but 0.2 per cent of the total intraerythrocytic K will increase serum [K] by 0.2 mEq/l.; and this rise in serum [K] will account for an apparent fall in $R_{sf}K$ from 0.96 to 0.92. It will be noted that the difference in the K and Na distribution ratios determined here is of this same order.

The observations of Greene *et al.* support the suspicion that leak of K may account for the apparent depression of the distribution ratio for K. These investigators found a mean $R_{sf}K$ of 0.75 in man; using the same technics they observed the ratio to be 0.94 in dogs (7). The [K] in the erythrocyte of the dog is approximately one-twelfth that of man (11). There would, presumably, have been escape of less K from the dog's red cell and this may well have underlain the observed discrepancy.

3. *Protein 'binding'.* The data reported here might be interpreted to indicate that a small portion of the K in serum is not free to diffuse, perhaps because it is rendered inactive or 'bound' by serum proteins. Ingraham *et al.* have studied the distribution of K and other ions between dog's plasma and its ultrafiltrate produced across a collodion membrane (10). Experimental variations in pCO_2 exerted on the plasma to alter pH from 7.0 to 7.7 produced a fall in mean $R_{sf}K$ from 0.90 to 0.82, and in mean $R_{sf}Na$ from 0.93 to 0.91. These changes in degree of diffusion resulting from changes in pH were attributed to alterations in cation binding by the serum proteins.

The explanation, therefore, of the small discrepancy in the data reported here between $R_{sf}K$ of 0.92 and $R_{sf}Na$ of 0.96 is uncertain. In any event, the probable error introduced by the escape of K from cells to serum may well account for the larger part of the difference. Under these circumstances the amount of K which is 'bound' by serum proteins becomes virtually insignificant in terms of presently available analytical technics.

If this interpretation is correct, it would appear proper to assign K to the same category as Na, Cl, and HCO_3 and, for practical purposes, consider it to exist in a freely diffusible state in serum and to distribute itself between serum and interstitial fluid in consonance with the Gibbs-Donnan equilibrium (13). From these considerations it is concluded that, if due care is exercised to minimise the transfer of K from erythrocytes to serum, a measure of serum [K] provides a useful estimate of interstitial fluid [K].

SUMMARY

1. Distribution ratios for K and Na have been determined in 22 instances in man by analysis of serum and of pleural, peritoneal and subcutaneous fluids.

2. Mean $R_{sf}K$ was 0.92 (range: 0.82-1.03) and mean $R_{sf}Na$ was 0.96 (range: 0.91-1.00).

3. Under the circumstances of sampling blood there is ample opportunity for the transfer of K from cells to serum sufficient to lower the observed ratio from that indicating free diffusibility (0.96) to that determined here (0.92).

4. K would appear, therefore, to exist in a freely diffusible state in serum in terms of presently available analytical technics, and the distribution of K between serum and interstitial fluid is consonant with the values predicted by the Gibbs-Donnan equilibrium.

We are deeply indebted to Dr. John W. Berry for his generous advice in the construction of our apparatus and to Mrs. Martha Jaffe for her valuable technical assistance.

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BILE ACIDS AND THEIR CHOLINE SALTS APPLIED TO THE INNER SURFACE OF THE ISOLATED COLON AND ILEUM OF THE GUINEA PIG

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THE widespread notion that bile salts have a laxative action has never been proven convincingly. Glaessner and Singer (1) have shown that rectal introduction of bile causes prompt defecation. Schlüpbach (2) found no definite effect of bile on the movement of a Vella fistula in the dog. Application of bile to the serosa of the rabbit's intestine *in situ* caused inhibition, while the isolated large intestine of the cat was stimulated. Errico (3) and Berti (4), by application of bile to the outside of the intestine, obtained inhibition, while rectal introduction stimulated activity. Ott and Scott (5), working with rabbit's and cat's gut *in situ* and *in vitro*, reported variable results in either case.

Application of agents to the serosa of the gut does not correspond to physiological conditions. We have shown (6) that physiological agents, applied to the isolated intestine, have a different action whether applied to the serosa or mucosa. Boulet (7), applying bile to the inside of isolated human and animal intestines, obtained variably inhibition and stimulation. Unfortunately, his preparations were not taken fresh from the living organism and had lost much of their original sensitivity, as is evidenced by the high inside pressure required to elicit peristalsis.

In the present investigation various bile acids at varying concentrations were applied to the inside of the intestine, using the distal colon and the ileum of the guinea pig. We also tested the choline salts of the same bile acids. Choline cholate and desoxycholate have been prepared before by Glücksmann (8). It is claimed in his patent publication that these compounds, given orally, exert a laxative action due to the preservation of the efficacy of choline, otherwise lost in oral medication. No evidence is presented for this claim.

MATERIAL AND METHODS

The substances investigated were choline chloride, cholic acid, desoxycholic acid, purified bile acids consisting of the natural mixture of glycocholic and taurocholic acid as obtained from ox bile; choline cholate containing 27.5 per cent choline calculated as chloride; choline desoxycholate containing 27.5 per cent choline; choline salt of bile acids containing 22.5 per cent choline.

Dehydrocholic acid was not used, because its solubility at the pH of Tyrode solution is too low. The choline salt of that acid could not be obtained.

The apparatus and procedure are described in our previous paper (6). In the present work, we investigated also the effect of washing the gut after treatment by emptying and refilling with Tyrode solution. The washing of the colon offered no

Received for publication March 12, 1948.

difficulty, but caution was necessary in the case of the ileum because the moving fluid easily stimulated it into spastic contractions at the lower outlet and caused distention of the upper part. The material was dissolved in Tyrode solution and the pH readjusted to 7.3-7.4. The quantity of medication in each case was diluted to 1 cc., injected through the rubber tubing leading to the gut, and washed into it with .5 cc. of Tyrode solution.

Sixty-five guinea pigs were used, each pig supplying one strip of distal colon and two or three of ileum.

RESULTS

Peristaltic oscillations (p)¹ of the colon usually begin at the low inside pressure of 1-2 cm. The p contraction begins at the upper end and travels down while the gut contracts longitudinally. This is followed by relaxation in both movements. Therefore, both levers move in the same direction. This coincidence of waves, however, does not apply to the amplitude, in which respect opposite effects are often observed. With increase in the t_l , the p waves are often considerably diminished while the l waves may increase. Decreased registration in p waves does not necessarily mean rest or relaxation. At high t_l small peristaltic waves may follow in quick succession, without causing propulsion of liquid, and therefore this activity is not adequately shown in the record.

The quick succession of small waves corresponds to overstimulation. A drop in t_l , as obtained by washing, causes a slowing in the rate of the waves.

A peristaltic wave in the ileum begins in most cases with a slight longitudinal contraction; then, while p travels down, the gut lengthens. Therefore, both levers move in opposite directions. A maximally stimulated t_l may coincide with a relaxed t_p , or an increased t_p may temporarily extend over the whole length of the gut.

The small intestine only exceptionally gives a continuous oscillation. As a rule, there is a group of waves followed by a period of rest at moderate t_l . Such wave groups may be elicited by outside stimuli. Immediately after one wave group, the sensitivity is low, the longer the rest period, the larger is the amplitude and number of following contractions. If t_l increases, the rest period becomes longer until finally the gut is without movement at high t_l and low t_p . The responses produced by the various substances are presented in the table and illustrated in figures 1 to 8.

Figure 9 shows the stimulating effect of choline bile acid salt on a colon strip that had been left without oxygen for 15 minutes and hence performed poorly.

Intestinal strips maximally shortened after application of any of the choline compounds failed to relax if $\frac{1}{2}$ mgm. of papaverine-HCl (which gives with Tyrode solution at pH 7.4 an opalizing solution of papaverine base) was placed inside the gut. The same dose, given to the outside bath of 250 cc. caused immediate maximal relaxation.

All compounds exerted a stimulating effect on the intestine after application to the mucosa although not always in identical reactions. High t_l not only offers resistance to l but also to the p -movements manifested by a slowing of the waves, and—predominantly in the ileum—by ultimate arrest. The intestine in high tonus

¹ Abbreviations used: longitudinal tonus— t_l ; peristaltic (circular) tonus— t_p ; longitudinal waves— l ; peristaltic waves— p .

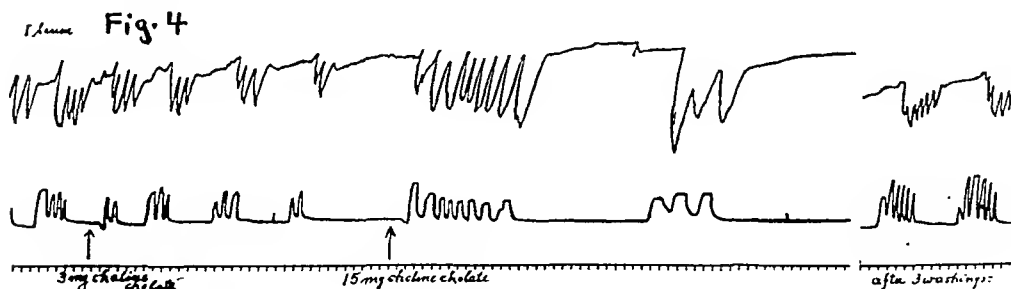
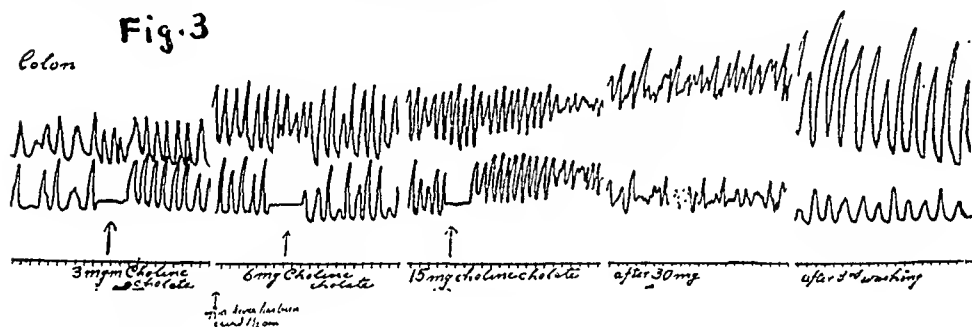
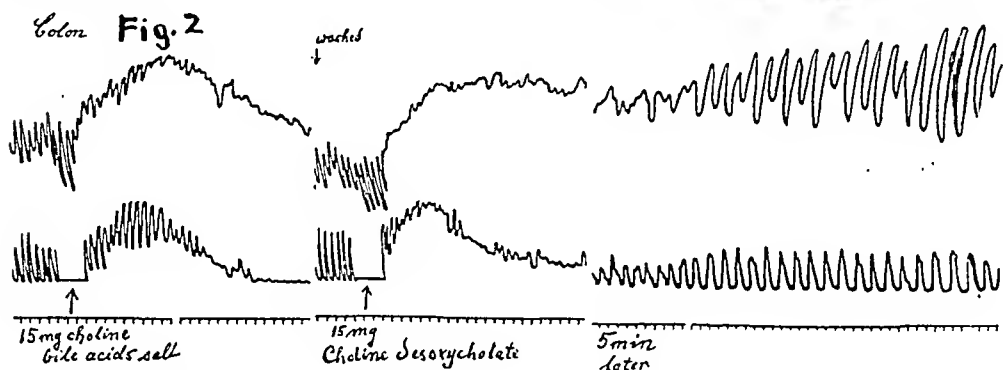
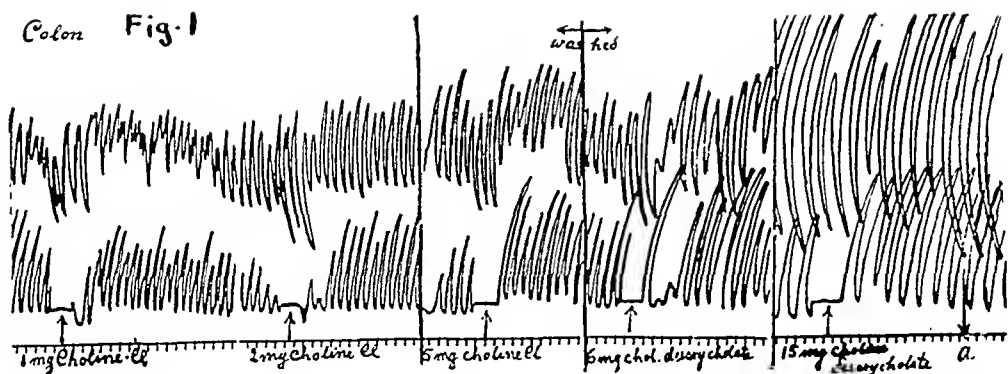


FIG. 1-4. Time interval, 10 seconds; upper tracing, longitudinal movements; lower tracing, transversal movements

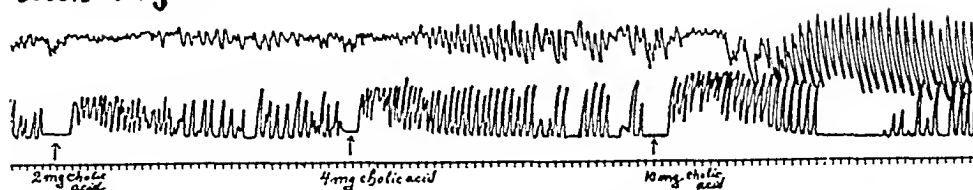
Fig. 1. STIMULATION OF COLON by choline. Drop in longitudinal tonus after washing and enormous stimulation by choline desoxycholate. The upper lever was lowered twice at 'a' to keep the tracing on the paper. This compensated in the tracing for the tonus increase. Time between sections of record, 3 to 5 minutes.

Fig. 2. TEMPORARY TONUS INCREASE by choline bile acids salts, as compared with permanent increase and slow large waves caused by choline desoxycholate.

Fig. 3. STIMULATION AND TEMPORARY ACCELERATION of colon by choline cholate, inhibition by higher doses and effect of washing. After 3 mgm. upper lever had to be raised to prevent overlapping of tracings since there was a relaxation of t_1 .

Fig. 4. EFFECT OF CHOLINE CHOLATE on ileum and effect of washing. Note group movements.

Colon Fig. 5



Ileum Fig. 6

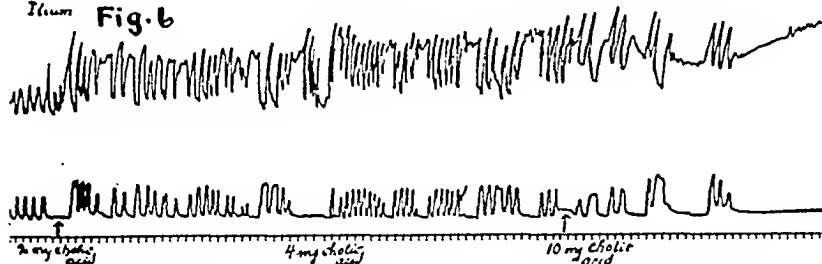
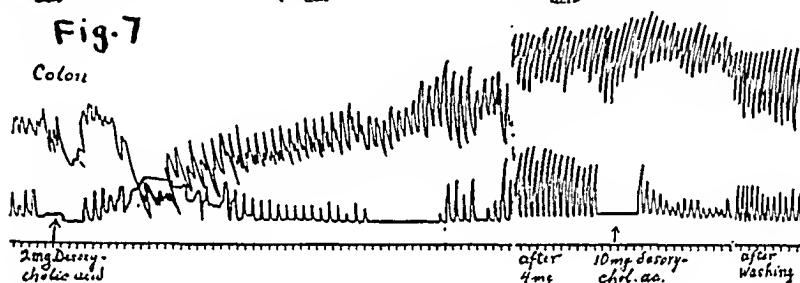
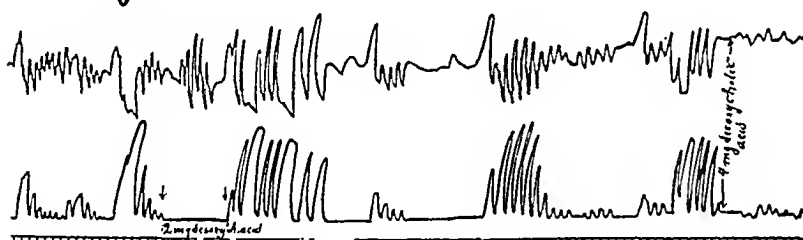


Fig. 7

Colon



Ileum Fig. 8



Colon Fig. 9

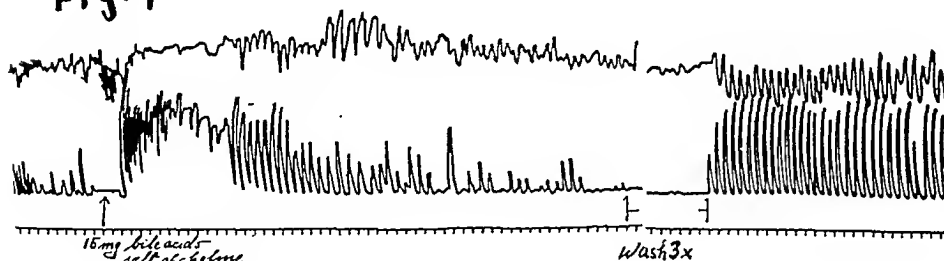


FIG. 5-9. Time interval, 10 seconds; upper tracing longitudinal movements; lower tracing, transversal movements

Fig. 5. STIMULATING EFFECT OF CHOLIC acid on colon. Absence of tonus increase.

Fig. 6. STIMULATION OF ILEUM by cholic acid; 10 mgm. caused tonus increase and inhibition.

Fig. 7. DESOXYCHOLIC ACID on colon. Stimulation with tonus increase. Regularizing effect of washing.

Fig. 8. DESOXYCHOLIC ACID on ileum; 2 mgm. stimulation; 4 mgm. inhibition.

Fig. 9. See text.

TABLE 1

SUBSTANCE	DOSE	t_l	t_p	l RATE	l AMPL.	p RATE	p AMPL.	EFFECT OF WASHING
<i>Colon</i>								
Choline chloride	mgm. 1-2	Inc.	Pass. inc		Inc.		Inc.	
	5-10	Add. inc.		Dec.	Dec.	Dec.	Dec.	T_l Dec.; l and p recover.
Desoxycholic acid	2-4	Mod. inc.	Pass. inc.		Inc.		Inc.	
	up to 30	Mod. add. inc.		Dec.	1. St. inc. 2. Sl. dec.	Dec.	1. St. inc. 2. Sl. dec.	T_l unchanged. l , p Sl. dec. Very regular
Choline desoxycholate	3	Inc.	Pass. inc.		St. inc.		St. inc.	
	15-30	Max. inc.		Sharp dec.	St. inc.	Dec.	1. inc. 2. Dec. or cess.	Rate recovers ampl. dec.
Bile acids	12.5	Inc.		Dec.	Inc.	Dec.	Inc.	
	25	Dec. no ch.		Irreg.	Dec.	Irreg.	Dec.	Activity inc.
Choline salt of bile acids	3-15	Inc.			Add. inc.		Add. inc.	
	15-30	1. Inc. 2. Dec.	Pass. inc.	Dec.	Inc.	Dec.	1. Inc. 2. Dec.	l and p large regular.
Cholic acid	2, 4, 10	Dec. no ch.	Pass. inc.		Add. inc.		Add. inc.	
	20	Mod. inc.			Inc.		Inc.	Ampl. of l and p dec.
Choline cholate	3	No ch., sl. dec.	Pass. inc.	Inc.	Inc.	Inc.	Inc.	
	6, 15, 30	Inc.	Inc.	Dec.	1. Inc. 2. Dec.	Dec.	1. Inc. 2. Dec.	Partl: inc. l & p Contd: Dec.
<i>Ileum</i>								
Choline chloride	2	Inc.			Inc.		Inc.	
	5-10	Pass. inc.	Sp.	St. dec.	Dec. or cess.		Dec. or cess.	T_l dec. gut relaxed, no waves
Desoxycholic acid	2				Inc.		Inc.	
	4	Mod. inc.			Dec. or cess.		Dec. or cess.	T_l high; waves, if abolished, re-stored.
Choline desoxycholate	3	Inc.		Dec.	Inc.	Dec.	Inc.	
	6	Add. inc.		Add. dec.	Add. inc.	Dec.	Inc.	
	over 6	High	Inc.	Cess.				Relaxed, inactive

TABLE 1—Continued

SUBSTANCE	DOSE	<i>tl</i>	<i>tp</i>	<i>l</i> RATE	<i>l</i> AMPL.	<i>p</i> RATE	<i>p</i> AMPL.	EFFECT OF WASHING
<i>Ileum—Continued</i>								
Bile acids	mgm. 12.5	No ch.			Inc.		Inc.	
	25	No ch.	Sl. pass. inc.		Dec. or cess.		Dec. or cess.	Waves are restored.
Choline salt of bile acids	3	Inc.	Spastic	Dec.	Pass. inc.	Dec.	Pass. inc.	Motionless, relaxed.
Cholic acid	2, 4	Add. mod. inc.			Inc.		Inc.	
	10	Add. inc.		Dec.	Dec. or cess.	Dec.	Dec. or cess.	Motionless.
Choline cho- late	3 or more	Inc.		Dec.	Inc.	Dec.	Inc.	
	10	Add. inc.	No ch.	Dec.	Dec.	Dec.	Dec.	
	15	Add. inc.		Either cess. of <i>l</i> and <i>p</i> with occasional slow large waves or superficial quick waves				Motionless.

Abbreviations

Inc. —increase
Dec. —decrease
Add. —additive
Mod. —moderate

Sl. —slight
Pass. —passing
St. —strong
Irreg. —irregular

Cess. —cessation
Sp. —spastic
1. —first response
2. —later response

is also less ready to respond with waves to incidental stimuli. The fact that in the small intestine both muscle layers work in the opposite direction causes some mutual inhibition of wave movements when the tonus of both layers increases as occurs after application of choline compounds. In the large intestine where both muscles work in the same sense, no interference exists and consequently complete arrest obtains only by maximal stimulation of tonus.

Among the compounds tested, the various bile acids have more of a stimulating effect on the rhythmic contraction than on the tonus, although this latter effect is always present at high doses. The mixture of conjugated bile acids seems to be the least efficient; while desoxycholic acid is the most active. The early inhibiting effect of the latter on the small intestine at relatively small tonus increase shows that the inhibition in the ileum is not exclusively a matter of tonus. Cholic acid shows a feature not observed in the other compounds—namely, a decrease of the longitudinal tonus of the colon at low dosage coinciding with a temporary *p* increase. Tonus increase with larger doses is not as high as with desoxycholic acid.

Choline chloride proved to be a powerful stimulant of both tonus and oscillatory movements with an early appearance of the competitive effects of both tendencies. While some intestinal strips showed extreme rhythmic movements after moderate doses, others were at rest in spastic contractions of both layers. Washing-out left such segments relaxed, as of exhaustion. The choline-bile acid compounds showed a combination, or moderation of both effects. Due to an increased stimulation of the oscillatory movements, the arrest of *l* and *p* by high tonus was delayed, or prevented altogether.

The tonus increases with bile acid-choline salt are not sustained, the gut tends to become inactive (fig. 2). Choline cholate shows the unusual case of a frequency stimulation in the colon at low dosage. This is a parallel to the drop in t_1 by cholic acid. At high dosage it increases the tonus considerably and slows the rhythmic movements. After choline desoxycholate, which is the most powerful agent, the oscillations in the colon seldom really cease, but are transformed into very slow waves of large amplitude. In the small intestine, tonic arrest may occur.

After partial washing following these choline compounds, the gut usually shows strong rhythmic activity, while prolonged washing causes relaxation.

In comparing our investigations with those of Boulet it appears that the concentrations applied by him were considerably higher than those in our experiments. In the light of our results, his intestinal preparations were first stimulated, then brought to spastic rest and finally to relaxation by fatigue. The concentrations of bile salts in bile are decidedly higher than those employed by us, but the factor of physiological dilution in the intestine is unknown.

From our experiences, we conclude that bile has a regulating influence on intestinal activity, but it is not justified to ascribe to bile an outspoken laxative effect, because large doses would rather lead to tonic arrest of peristalsis than to excessive activity.

SUMMARY

Conjugated bile acids, desoxycholic and cholic acid, choline, and also the choline salts of these bile acids exert analogous influences on the small and large isolated intestine of the guinea-pig, when applied to the internal surface. Low doses always cause stimulation of the rhythmic movements. With higher doses, the tonus increases and tends to counteract the wave movements. This is expressed by slowing of the rate and later by decrease of the amplitude of the waves. Bile acids stimulate more the oscillatory movements; choline has a stronger influence on tonus increase.

The choline salts of bile acids combine and moderate both effects. Among the bile acids, the conjugated natural acid mixture is of weakest action, cholic, stronger and desoxycholic acid, strongest. While choline finally tends to produce spastic arrest of movements, the choline salts of bile acids mostly maintain a slow longitudinal and peristaltic movement even at high tonus. The small intestine more readily is subject to tonic arrest than the large intestine. Washing out of the treated preparation decreases the tonus and establishes rhythmic movements mostly of greater regularity than is observed in the untreated gut. Prolonged washing leads to arrest in relaxation.

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THE AMERICAN JOURNAL OF PHYSIOLOGY

Published by
THE AMERICAN PHYSIOLOGICAL SOCIETY

VOLUME 153

June 1, 1948

NUMBER 3

EFFECT OF NEPHRECTOMY IN THE EVISCERATED RAT UPON TOLERANCE FOR INTRAVENOUSLY ADMINISTERED GLUCOSE

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EVISCERATED rabbits (1) and rats (2) show an increase in tolerance for intravenously administered glucose when the kidneys are removed. Reincke (3) has presented strong evidence that the kidneys of the rat can form some glucose from non-carbohydrate sources.

In the present study eviscerated and eviscerated-nephrectomized rats were given continuous intravenous infusions of glucose with and without insulin for periods of 2 and 24 hours. At the end of 2 hours the average values for blood glucose were significantly lower in the nephrectomized-eviscerated rats than in the non-nephrectomized-eviscerated rats at each glucose load with and without insulin. Within the 24-hour period the nephrectomized-eviscerated rats tolerated a higher glucose load than the non-nephrectomized-eviscerated rats at each glucose load when no insulin was given and when the insulin dose was small. When the comparisons were made at a high level of insulin dosage with high glucose loads the order of tolerance was reversed in that the nephrectomized animals had higher average terminal values for blood-glucose than the non-nephrectomized rats.

METHODS

Male rats of the Sprague-Dawley strain were fed Friskies Dog Cubes. At a weight of 185 to 205 grams, the inferior vena cava was ligated between the liver and the kidneys in order to cause the development of a collateral circulation. Asepsis

Received for publication May 5, 1948.

was preserved in this operation. When the animals reached a weight of 250 (± 2) grams they were anesthetized (intraperitoneal injection of 18 mgm. of cyclopentenylallyl-barbituric acid sodium) and eviscerated by the procedure of Ingle and Griffith (4). The kidneys were removed in the same operation. Hemostasis was attained by applying a gelatin sponge (Gelfoam, Upjohn) with thrombin to the stumps of the oesophagus, colon, ligated vessels and between the muscle and skin when the incisions were closed. The animals were not fasted prior to operation.

Intravenous injections of solutions containing 0.9 per cent sodium chloride and varying concentrations of glucose (C. P. Dextrose, Merck) with and without crystalline zinc insulin (Lilly) were made by two continuous injection machines which delivered fluid from each syringe at the rate of 20 cc. in 24 hours. The glucose load is expressed as mgm. of glucose per 100 grams of rat per hour (mgm/100/h). Syringes of the Luer-Lok type (Becton-Dickinson) were selected to deliver 20 cc. with a stroke of 65 mm. Six syringes were operated by each machine. The machines were powered by synchronous motors, and the reduction of motion was achieved by a precision-built system of gears so that an exact control of the rate of injection was attained.

The infusions were made into the saphenous vein of the right hind leg and were started within 5 minutes following the removal of the liver. The animals were secured in a supine position on an animal board and were enclosed in a cabinet with the temperature constant at 26.5° C. (± 0.5). The temperature of the room was maintained at 74° to 78° F. and the humidity at 30 per cent to 35 per cent of saturation.

The analyses of blood glucose were made by the method of Miller and Van Slyke (5). This method measures small amounts of non-fermentable reducing substances which accumulate in the blood of eviscerated rats. Samples of blood were taken from the jugular vein at the end of 2 hours of infusion in experiment 1, and at the end of 24 hours of infusion in experiment 2.

EXPERIMENTS AND RESULTS

In *experiment 1*, (fig. 1) 11 groups of eviscerated rats, having 12 pairs of rats in each group, were infused with glucose for a period of 2 hours. One rat of each pair was nephrectomized. Six groups represented glucose loads of 6, 10, 14, 18, 22 and 26/100/h without insulin. Five groups were given insulin at the rate of 4 units per 24 hours per rat and represented glucose loads of 64, 68, 72, 76 and 80/100/h. In each group the average level of blood glucose at the end of 2 hours was significantly lower in the nephrectomized-eviscerated rats as compared to the non-nephrectomized-eviscerated rats.

In *experiment 2*, (fig. 2) 9 groups of eviscerated rats having 12 pairs of animals in each group were infused with glucose for a period of 24 hours. One rat of each pair was nephrectomized. Three groups represented glucose loads of 4, 8 and 12/100/h without insulin; 2 groups represented glucose loads of 20 and 24/100/h with an insulin dose of 0.5 units per 24 hours per rat; and 4 groups represented glucose loads of 36, 40, 40 and 44/100/h with an insulin dose of 4 units per 24 hours per rat. In the groups without insulin and in those with an insulin dose of 0.5 units per 24 hours per rat the average level of blood glucose was lower in the nephrectomized-

eviscerated rats than in the non-nephrectomized rats. In the groups which received 4 units of insulin per 24 hours per rat and glucose loads of 36, 40 and 44/100/h the nephrectomized rats showed higher average values for blood glucose at the end of 24 hours than did the non-nephrectomized rats.

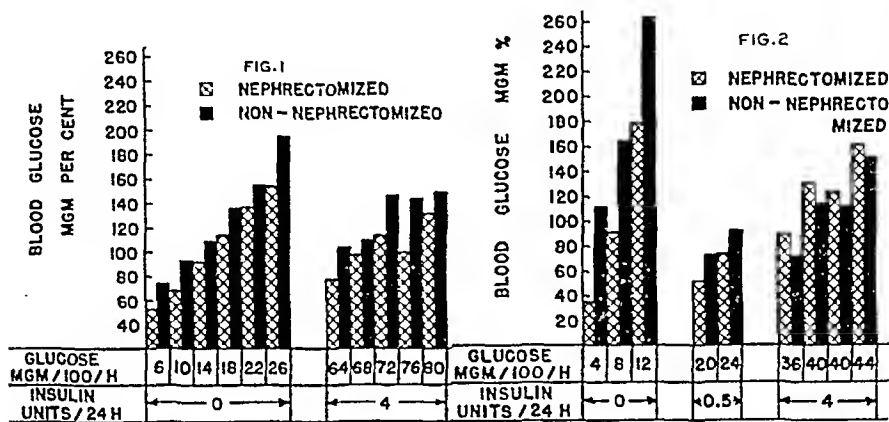


Fig. 1. EFFECT OF NEPHRECTOMY in the eviscerated rat upon the average level of blood glucose as related to insulin dosage and glucose load. Two hours.

Fig. 2. EFFECT OF NEPHRECTOMY in the eviscerated rat upon the average level of blood glucose as related to insulin dosage and glucose load. Twenty-four hours.

DISCUSSION

Immediately following the removal of the kidneys from the eviscerated rat, the tolerance for glucose is higher than in animals having the kidneys intact. We assume, on the basis of earlier work (3, 6), that some gluconeogenesis occurs in the kidney of the rat. However, when the glucose tolerance is brought near its ceiling by high insulin dosage the nephrectomized-eviscerated rat shows a greater decrease in tolerance for glucose by the end of 24 hours than similar animals having intact kidneys. This cannot be explained by the assumption that the non-nephrectomized rats excreted glucose. No urinary glucose was found. We suggest that this change reflects the more rapid approach to moribundity by the nephrectomized rat and represents a non-specific change which masks the rôle of the kidney in gluconeogenesis. This terminal decrease in tolerance for glucose by the nephrectomized-eviscerated rat resembles the change which occurs in the adrenalectomized-eviscerated rat (7). Although the tolerance for glucose is increased immediately following adrenalectomy these animals show a decrease in tolerance for a high glucose load by the end of 24 hours.

The differences in glucose tolerance between nephrectomized and non-nephrectomized-eviscerated rats can be covered by a change in glucose load of 4/100/h or slightly greater. This suggests, but does not prove, that the amount of glucose formed by the kidney under these conditions is small. The studies of Roberts and Samuels (6) indicate that renal gluconeogenesis is stimulated by fasting.

SUMMARY

Male rats (185-205 grams) were caused to develop a collateral circulation by ligation of the inferior vena cava. At a weight of 250 (± 2) grams the animals were

anesthetized (cyclopal) and eviscerated. The kidneys were removed from one animal of each pair. Infusions into the saphenous vein were made by continuous injection machines which delivered fluid at the rate of 20 cc. in 24 hours per rat. The level of blood glucose at the end of the injection period was the index of glucose tolerance.

In *experiment 1*, 11 groups of eviscerated rats having 12 pairs of rats in each group were infused with glucose for 2 hours. One rat of each pair was nephrectomized. At each glucose load with and without insulin the average level of blood glucose was significantly lower in the nephrectomized than in the non-nephrectomized animals.

In *experiment 2*, 9 groups of eviscerated rats having 12 pairs of animals in each group were infused with glucose for a period of 24 hours. In the groups without insulin and in those with an insulin dose of 0.5 units per 24 hours per rat the average terminal level of blood glucose was lower in the nephrectomized-eviscerated than in the non-nephrectomized rats. At the higher levels of insulin dosage and glucose loads the order of tolerance was reversed and the nephrectomized animals had higher average terminal values for blood glucose than the non-nephrectomized rats.

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EFFECT OF THIOURACIL ON LIVER REGENERATION

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THIS study was undertaken to determine the effect of thiouracil on the rate of regeneration of the rat liver. Based on the experiments of Paschkis and his co-workers (1, 2), who have shown that thiouracil exhibits an inhibitory or preventive effect on the induction of hepatic tumors by 2-acetamino fluorene or p-dimethylaminoazobenzene, we originally felt that thiouracil might inhibit the rate of liver regeneration. Our results, however, have shown the reverse to be true; that is, thiouracil administration enhances the rate of regeneration of the rat's liver.

While some studies have been conducted to determine the factors which affect liver regeneration, the relationship of thyroid function to this phenomenon has never been clearly demonstrated. Higgins (3) has presented evidence to show that there is an increased rate of regeneration of the rat liver associated with hyperthyroidism. This work, however, is open to some question as it took 14 days before any change in rate of regeneration between the two groups became obvious, and it was not until the fourth week that a 20 per cent increase in rate in the treated group was noted. Since, in the partially hepatectomized rat, regeneration is usually completed by the 14th day, we cannot assume that his experiments show an increased rate of regeneration. Rapport (4) has recently reported a study of the effect of thyroidectomy on liver regeneration and nucleic acid partition. He failed to come to any definite conclusions concerning the effect of thyroidectomy on liver regeneration. Drabkin (5) performed thyroidectomies in rats and followed this with partial hepatectomy 30 days later. He states, "liver regeneration was not strikingly interfered with, although it was of a smaller magnitude than normal."

A number of workers (6-8) have reported an increase in liver weight following thiourea or thiouracil administration. May, Moseley and Forbes (7) studied the effect of thiourea on body fat and liver glycogen in rats. They showed that thiourea does not affect the distribution of neutral fat in the rat liver and that its administration leads to a marked increase in liver weight as well as an increase in liver glycogen. Leatham and Seeley (8, 9) studied liver protein concentrations associated with thiouracil administration and found an increase in liver weight as well as an increase in total liver protein. These workers also performed thyroidectomies in an attempt to determine whether or not the increase in total liver protein associated with thiouracil feeding could be due to the hypothyroid state induced by the drug. They found that thyroidectomy does not bring about the increase in liver weight or alter the total liver protein as compared to thiouracil, thus suggesting another mode of action of the drug on the liver.

György, Rose and Goldblatt (10, 11) have determined the effect of various goitrogenic substances on experimental dietary cirrhosis. Their results indicate that both thiouracil and propylthiouracil have a preventive effect on liver injury when administered along with a cirrhosis-producing diet. They also present evidence of parallelism of activity between goitrogenic and preventive potency, since they found that propylthiouracil was most effective followed by thiouracil, thiourea and amino thiozole in order of descending anti-cirrhotic potency. They also claim, on the basis of unpublished data, that "thiouracil appeared to exert no effect or even a slightly deleterious one on a pre-existing cirrhosis."

Received for publication May 25, 1948.

¹ Aided by a grant from the National Advisory Cancer Council.

Handler (12, 13) has studied the influence of thyroid activity on liver lipids in choline and cystine deficient diets. Rats fed a high fat diet were concurrently fed either thyroid substance or thiouracil. Total liver lipids, liver cholesterol and phospho lipids were determined. The thiouracil-treated group showed an increase in liver lipids, while the thyroid-fed group showed a decrease in total hepatic lipids as compared to the controls. He also showed that following thyroidectomy or thiouracil administration there is a marked increase in cholesterol concentration and a small increase in neutral fat of livers of rats both on the control and choline-deficient diet. Thyroid feeding, on the other hand, caused a decrease in cholesterol concentration and neutral fat of the livers of animals both on a control as well as on a choline-deficient diet. He has concluded from this work that hypothyroidism causes an increase in cholesterol and neutral fat in the liver of rats, while hyperthyroidism causes a decrease of cholesterol and neutral fat in the liver.

Shipley, Chudzik and György (14) determined the effects of extirpation of the thyroid, adrenals, pituitary and gonads on production of fatty liver by dietary means. Liver fat was determined chemically after they had maintained the animals on a cirrhosis-producing diet for 21 days. The most striking results were obtained in the thyroidectomized group in which there was a marked decrease in liver fat as compared to the control, non-operated animals.

Hence, while there is a trend in thought today that thiouracil has an ameliorative effect on certain liver diseases and that a lowered metabolic rate plays an important rôle in this effect, the experimental evidence to substantiate this view is by no means conclusive.

METHODS

Sixty-four healthy male albino rats of the Harlan strain weighing from 200 to 305 grams were used as experimental animals. The animals were paired off into matched weights and divided into two groups. Both groups received *ad libitum* feedings of Purina fox chow blox. Animals in Group II, the treats, received daily intraperitoneal injections of 8 mgm. of thiouracil per 100 grams of body weight beginning on the day of operation and extending until necropsy on the 11th post-operative day. Thiouracil was dissolved in distilled water. Complete solubility was never attained, but the suspension was of small enough particle size to pass through a 22-gauge needle. The intraperitoneal route was chosen because of the necessity of controlling the dosage.

Partial hepatectomy was performed in the standard method described by Higgins and Anderson (15). Ether anesthesia was used. Cleanliness but no asepsis was employed. The wet weight of the liver removed was determined and the specimen then placed in a constant temperature oven at 100° C. until constant dry weight was obtained. This usually required from 72 to 96 hours. On the 11th post-operative day, the animals were killed by a blow on the head, body weight determined, and then necropsied. The total liver was then removed, weighed and dried to constant weight in the manner already described.

Higgins and Anderson (15), Brues (16), and Denton and Ivy (17) having shown that the amount of liver removed bears a constant ratio to the entire liver of the rat, the amount of liver remaining at operation may be computed. Since one may assume that 68.5 per cent of the total liver was removed, the amount remaining at operation is 31.5 per cent, or 46 per cent of the portion removed. The liver increment or mass regenerated was determined by subtracting the weight of the liver remaining at operation from the total regenerated liver removed on the 11th post-operative day. Only dry weights were used in the statistical analysis.

RESULTS

The incidence of mortality due to surgery alone was 2 per cent. Fifteen per cent of the control group and 25 per cent of the treated group died before completion of the experiment. The most common cause of death in both groups was pneumonia, with death usually on the fourth day. No remarkable gross pathological findings were present in the treated group.

Table 1 presents the data on 31 control animals and 33 treated rats which survived the experiment. Two significant findings are seen to emerge from the data, the loss of body weight and the absolute liver increment of the two groups. When

TABLE 1¹

	1	2	3	4	5	6
	BODY WT. AT OPERATION	BODY WT. AT NECROPSY	DRY WT. OF LIVER REMOVED	AMT. OF LIVER RE- MAINING	DRY WT. OF LIVER AT NECROPSY	LIVER INCREMENT
Group I, Controls, 31 rats....	254.3 ± 7.16	245.8 ± 6.98	2.058 ± 0.469	0.905	2.650	1.702 ± 0.320
Group II, Thiouracil- treated, 33 rats.....	253.3 ± 6.87	237.4 ± 10.29	1.952 ± 0.219	0.867	3.160	2.250 ± 0.525
Difference...	1.0	8.4				.548
<i>t</i>	0.565	1.67	0.369			4.01

¹ Values are the means with their respective standard deviations in grams. Dry weights were determined by drying at 100°C. to constant weight. Figures in column 4 were obtained by multiplying the dry weight of the liver removed by 0.46. Column 6 figures were determined by subtracting individual values of column 4 from column 5. Since the ratios of wet to dry liver weights of both control and treated groups were the same, only dry weights were used in the statistical analysis.

the *t* value is computed for the difference in the average loss in body weight between the control and treated groups (using the method of paired comparisons), a value of 13.6 is derived, which means that the increased body weight loss associated with thiouracil administration is of significance. When the *t* value for liver increment is computed, *t* = 4.0, which means that the probability of the increased liver increment in the treated group having occurred by chance due to sampling error is less than one in one hundred.

DISCUSSION

While the administration of thiouracil undoubtedly affects thyroid function, it is not presently possible to ascertain whether its action on the regenerating liver is due to this action on the thyroid. We are faced with two main hypotheses. Does decreased metabolism induced by thiouracil have an effect on the regenerating liver? Or does thiouracil have a direct effect on the liver distinct from its goitrogenic activity?

Evidence in favor of increased liver regeneration associated with a reduction in general metabolism is certainly scanty and inconclusive. While it is true that hepatic degenerative changes are commonly found in hyperthyroidism, no evidence is available to show that hypothyroidism *per se* may play a definite ameliorative rôle in association with hepatic dysfunction. While it is possible that a reduction in body metabolism might increase the amount of available protein for the liver by decreasing extra hepatic usage, it is also possible that protein utilization by the liver may be markedly decreased in an animal of low metabolic activity. The fact that despite thiouracil administration experimental dietary cirrhosis still occurs, even though at a lower frequency, may only mean that there is a delay in the pathologic process. In other words, thiouracil and decreased metabolism may not actually prevent cirrhosis, but only delay its onset.

The point then arises as to whether or not thiouracil has a direct effect on the liver. Handler's work certainly shows that it is not lipotropic. The fact, however, that thiouracil increases total liver weight, total liver protein, and the rate of regeneration of the liver—phenomena which most probably do not occur in association with a hypothyroid state produced by thyroidectomy—certainly points strongly toward a direct effect. There is some further evidence supporting this point. Fitzhugh, Nelson and Holland (18, 19) have recently reported an interesting finding associated with chronic thiourea administration. They fed thiourea in various concentrations to rats for a period of two years. In the groups fed thiourea in a concentrate of 0.25 per cent or less for a period of at least 96 weeks, half the surviving animals had liver tumors. The tumors, some as large as 3 to 4 inches in diameter, were all benign hepatomas on microscopic examination. There were no metastases, and cirrhosis was minimal. This remarkable finding certainly leads one to think of a direct stimulating effect of thiouracil on the growth of the liver. The fact that cirrhosis was minimal in association with these tumors might also aid in explaining some of the discrepancies between the effect of thiouracil on liver fat and cirrhosis. It apparently is not cirrhosis-producing, even though it may possibly increase liver fat.

Thiouracil might also conceivably act by making available material for nucleic acid synthesis. The pyrimidine bases taking part in the structure of nucleic acids are derivatives of uracil. Cytosine and uracil are detectable in the urine of dogs fed the uracil. Counter evidence, though not conclusive, bearing on the utilization of thiouracil in the synthesis of nucleic acids, is the work of Plentl and Schoenhemier (20) in which they fed isotopically labelled uracil and found there was no incorporation of the compound into the nucleic acid of rats.

SUMMARY

Evidence is presented which shows that thiouracil, when administered to partially hepatectomized rats, increases the rate of regeneration of the liver. No definite conclusions can be drawn concerning the mechanism of the effect.

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EFFECTS OF ADDED CALCIUM UPON THE INTACT, BLOOD CIRCULATED, TURTLE HEART

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EFFECTS of calcium upon the physiology of the heart have been reported in many publications, as changes in calcium content or form alone, or in relation to other cations. The older studies, principally upon cold-blooded hearts, have been reviewed in a monograph by Clark (1). A critical review by Berliner (2) which includes mammalian studies and clinical trials has largely completed the subject through 1933. More recent studies and older ones that are particularly pertinent to the experiments of this paper will be cited.

The augmentation of cardiac contractility by adding calcium salts and the limitations to this effect have been explored extensively (1, 2). By contrast reports upon the effect of calcium on excitability of heart muscle are meager. Schultz (3), working with strips of turtle ventricle, found that in Ringer's solution with experimentally varied low concentrations of calcium an increase of calcium ions increased contractility and lowered excitability to induction shocks. Lussama (4) reported that frog hearts in a fluid containing one half of the normal calcium concentration exhibited enhancement of excitability and that fluid with twice the normal calcium concentration diminished excitability. The excitability story is not a clear one, however, due to the repeated observation that auricular and ventricular ectopic systoles, heterotopic arrhythmias, and tachycardias for brief periods can be produced by the administration of excess calcium (5, 6). These reactions give the appearance of increased excitability, seeming to contradict the evidence from electrical stimulation.

In studies on the excitability of tissues other than heart muscle, i.e., frog nerve (7), skeletal muscle and neuromuscular junctions (8), it has been found that reduction or absence of calcium ions in the saline medium enhances excitability as measured by electrical thresholds. Increase of calcium ions in calcium-deficient saline raises the threshold (galvanic) of nerve according to a curve which is steep in the lower concentrations and flatter within a range between 1 and 2 millimolar CaCl_2 (cold-blooded Ringer's solution contains 0.012 per cent $\text{CaCl}_2 = 1.1$ millimolar). The increase in threshold is small in solutions above 2 millimolar until very high concentrations are reached where significant increases again occur (9).

Addition of calcium ions to the medium bathing nerve does not excite a spontaneous discharge of impulses as it often does in heart muscle, but spontaneous discharges are produced in nerve by reduction of calcium, and the lower the concentration of calcium ions, the greater the frequency of spontaneous discharges (10). The addition of calcium ions reduces or abolishes them. Thus, no excitability paradox is encountered in comparing the effects of calcium ions upon electrical thresholds and upon spontaneous discharges in nerve. Depression by added calcium ions is indicated by both kinds of observations.

In two reported clinical cases, the administration of CaCl_2 has had the effect of suppressing ectopic ventricular discharges (11). Isolated mammalian auricular strips made active by histamine had the spontaneous activity abolished by calcium salt, and the development of spontaneous rhythmicity could also be prevented by it (12). In these two reports, which are different from all others,

Received for publication January 26, 1948.

¹ Traveling Fellow of the Rockefeller Foundation, 1947, from Zagreb, Yugoslavia.

the effects of added calcium ions upon ectopic activity of heart muscle were similar in direction to the effects of added calcium ions upon spontaneously discharging nerve fibers.

This study was planned for the gathering of more precise information upon the reactions of cardiac tissues in situ, nourished by blood, to the administration of calcium ions. It was especially hoped that the observations would make possible an explanation of the apparent disagreement between the excitability changes indicated by electrical thresholds and those which can be deduced from the induction of spontaneous rhythms in auricular and ventricular muscle.

PROCEDURES

P. elegans turtles weighing 900 to 1150 grams were used in all experiments. The brain was destroyed by thoroughly crushing the head. A circular portion of plastron 3 inches in diameter was removed by a power-driven saw, exposing the heart.

For kymographic recording of the relative changes in force of contraction, the ventricular apex was attached to a heart lever. A thread extended from the apex almost horizontally toward the caudal end of the animal, then passed under a pulley and turned vertically to be attached to the recording lever. A tension of 50 grams was added by hanging a weight on the lever. This was found to be sufficient to render any changes in tension due to variations in filling a negligible fraction of the total, and prevent changes in height of contraction from this cause.

Electrocardiograms were recorded by a G. E. Victor electrocardiograph. The method of leading to obtain sinus, auricular, and ventricular deflections is described in connection with the results.

For testing thresholds an electronic stimulator was used. The shocks delivered were almost entirely monophasic and had an over-all duration of 3 msec. The apparatus could be used as a self-excited oscillator, or the discharge could be triggered off when desired in order to deliver the shock at the chosen moment in the cardiac cycle of the driven heart. The S-A junction was crushed, and the heart was driven at a constant rate by induction shocks applied to the right atrium.

RESULTS

Contractility. The effects of varied doses of CaCl_2 upon the size of the excursion and upon tonus (contraction remainder in diastole) of the ventricular muscle are seen in figure 1. This is a series of records from the heart of a 1070 gram turtle. All doses were injected into the sinus venosus in this experiment. Results were somewhat more constant by this route than by vein.

With all doses except the strongest, CaCl_2 administration produced an increase in the height of the contraction summits above the control base line. However, this resulted in increased magnitude of excursion in only those trials in which the dose was not sufficiently large to increase tonus. Figure 1A illustrates the effects of a small dose (0.5 cc. of 1.5% CaCl_2). It shows one large contraction and a slight rise of tonus for two beats following injection. Such preliminary increases of contraction with an increase of tonus for one to three beats was a fairly constant feature. The events upon which most attention was centered were those which followed. In this record they consist of a gradual increase in the height of contractions without an in-

crease in tonus. This resulted in an increased magnitude of excursion which reached its maximum about 30 to 40 seconds after the injection and subsided within about an equal period. The total duration of increased contractility was little more than one minute.

The effects of a larger dose (0.3 cc. of 10% CaCl_2) are seen in figure 1B. After the preliminary effects, the heights of the summits and the tonus level were increased simultaneously. The rise of tonus was greater than the rise of the summits, therefore the excursion was reduced. The increased heights of the summits and of tonus reached their maxima within about 30 seconds after the injection and subsided more

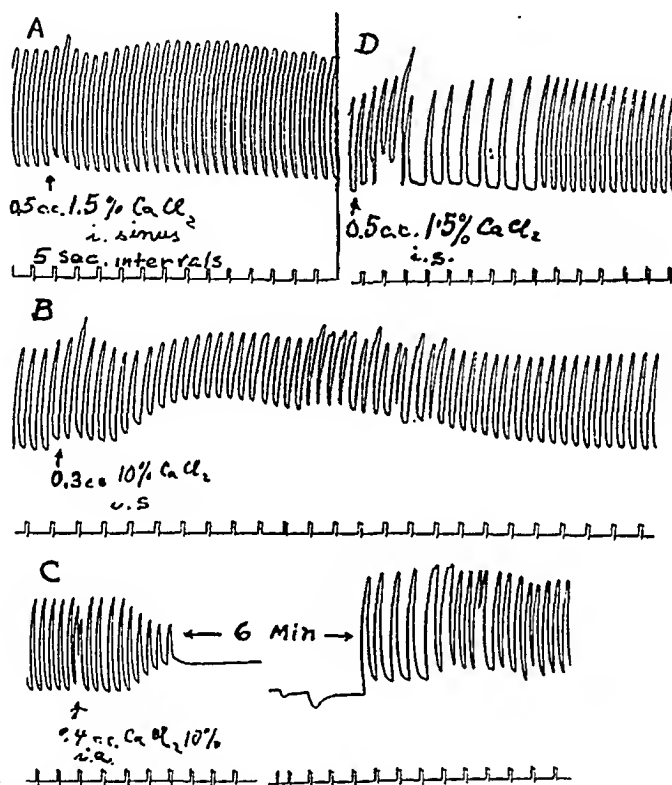


Fig. 1. CHANGES IN CONTRACTILITY and tonus following intra-sinus administration of CaCl_2 . Time intervals 5 sec. Description in text.

slowly, approaching the control levels in about $1\frac{1}{2}$ to 2 minutes. Auricular ectopic systoles occurred during a period of about 25 seconds during recovery from the summit height and tonus maxima.

The effects of a dose (0.4 cc. of 10% CaCl_2) large enough to stop the heart are shown in 1C. Here, with the rise in tonus there is a falling off in summit height during a period of about 10 seconds before contractions stopped. The atria and ventricle stopped in a condition of heightened tonus 25 seconds after injection. The first returning beats of the atria occurred almost 6 minutes after injection, and ventricular beats returned 23 seconds after the atria. This record shows clearly that during the time that the heart was stopped in calcium hypertonus² the ventricular

² The term 'calcium rigor', perhaps, should be reserved for the irreversible state of shortening observed by Howell (13) in ventricular strips in solutions with excess calcium.

muscle was by no means maximally contracted, though to visual observation both of the atria and the ventricle appeared to be in full contraction. The degree of shortening at first was about 35 per cent of a control contraction. During the 6 minutes of standstill this tonus shortening gradually disappeared. When the ventricle resumed activity there was 2:1 block for a few cycles, an ectopic atrial systole and then a return to regular rhythm. Upon return the ventricular beats were appreciably larger than the controls during the slow 2:1 block period and slightly larger for a few beats after the return to normal rate.

Other records of calcium hypertonus exhibit changes which, in general, were similar to those illustrated, but the quantitative variations were great. In one experiment the maintained shortening was 75 per cent of the control contraction height initially, and there was some elevation when rhythmicity returned. Calcium standstill in hypertonus consists of a submaximal maintained shortening combined with absence of discharges of the automatic excitatory mechanisms and failure to respond to driving stimuli.

Record 1D, made at the end of a long series of injections, shows the effects of a repetition of the dose used in the first trial, 1A. Comparison of 1D with 1A shows that after a larger preliminary reaction in 1D there was a staircase-like increase in height of contraction which in outline closely resembled that in 1A, though slightly more pronounced. In 1D there was a 2:1 block during the rise to maximum, but the smooth curve traced by the summits during block and after its disappearance indicate that the block did not affect the height of contraction. This is evidence that the added tension substantially eliminated the effects of filling (greater interval) upon contraction height.

Rhythmicity and Sino-Atrial Conduction. The kymograph records of numerous early experiments showed that important changes in rhythm resulted from administration of CaCl_2 . In order to study these changes with precision, electrocardiograms were made with one lead on the sinus venosus and the other in contact with the contents of the abdominal cavity a short distance caudad from and beneath the left side of the ventricle. Records made in this manner contain a distinct sinus deflection (labelled O) followed by the atrial and ventricular deflections. Short segments of record were taken at frequent intervals. A few of these, showing significant stages in the slowly changing effects, are reproduced.

Figure 2 illustrates the electrocardiograms obtained and the alterations induced by addition of CaCl_2 . 2A is a normal record showing the sequences of electrical activity in the sinus (O), atria (P), and ventricle (QRST). When the rate was rapid, as in this record, O came within the S-T interval. Record 2B, made immediately after injection of 0.2 cc. of 10 per cent CaCl_2 , shows no change in rate, rhythm, conduction interval or duration of electrical systole. There is a change in the form of S-T-T which will not be considered in this presentation. 2C made 9 minutes after injection shows practically no change in rate, but some shortening of the O-P interval. The P-R interval and duration of electrical systole were still unchanged. Figure 2D shows complete sino-atrial (O-P) dissociation and a change in the form of P which was to be expected with the advent of the ectopic atrial pacemaker. The rate is slower also. Duration of electrical systole was increased as expected with the slower

rate. This record was made 52 minutes after injection. The first evidence of O-P dissociation had appeared in earlier records about 25 minutes after injection.

After about three hours following the injection, the sinus seemed to have resumed the rôle of pacemaker during a part of the time. Record 2E might be so interpreted, though there is considerable variation in O-P intervals. In some of the records made at this time O followed P or QRS, but it was difficult to decide whether or not retrograde conduction existed.

A second injection of 0.3 cc. of 10 per cent CaCl_2 was made $3\frac{1}{2}$ hours after the first. The sinus ceased to beat for a short time. Record 2F, made immediately, contains no O deflections. They returned quickly, as is shown by the presence of independent O-waves in 2G, made one minute later. This record also contains evidence of a great variation in cycle length. During the whole period, since the begin-

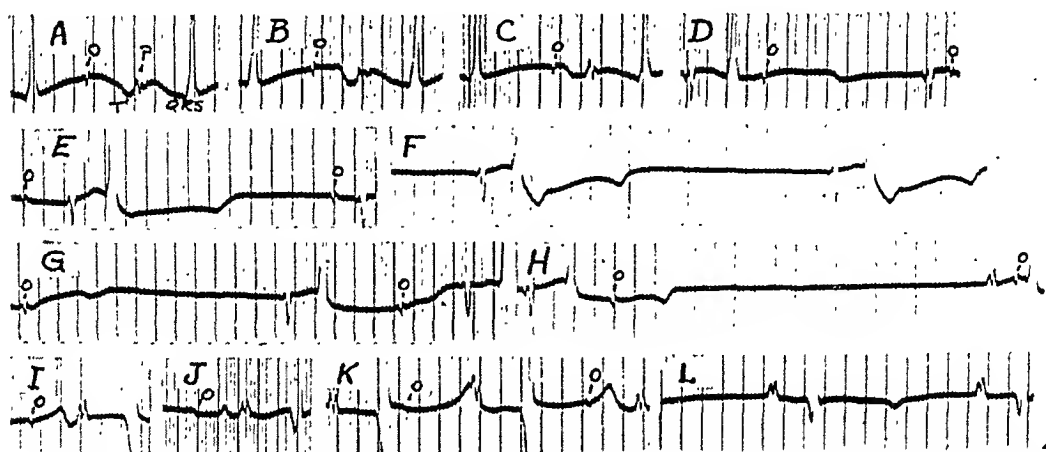


Fig. 2. ELECTROCARDIOGRAMS OF THE TURTLE, made so as to include the sinus deflections, labeled O. Effects of CaCl_2 described in text.

ning of O-P dissociation, there had been marked changes in rate with occasional coupling. Record 2H illustrates a change in form of P, indicative of a shifting atrial pacemaker which sometimes accompanied the change in rate. All of the above-described records were from a turtle with intact vagus nerves. However, the heads of all turtles used were well crushed, and it is highly probable that every one was effectively vagotomized centrally. To be sure of eliminating vagal influence, the vagi were sectioned in some turtles. Records from one of them are reproduced as figure 2I, J, K, and L. 2I and 2J are control records before and after section respectively. Measurements of rate from longer sections show no change. Record 2K, 8 minutes after administration of 0.2 cc. of 10 per cent CaCl_2 , shows the beginning of sino-atrial dissociation. Four hours later a second injection was made. Five minutes after this addition of 0.4 cc. of 10 per cent CaCl_2 , the sinus stopped for a short time, or was extremely slow and irregular. Record 2L contains no O deflections. Thus it is evident that the slowing and stopping of the sinus, the sino-atrial dissociation, and arrhythmias of atrial origin following CaCl_2 administration are not due to vagal effects.

In our experiments no auricular standstill with idioventricular rhythm nor A-V dissociation has been observed, though 2:1 A-V block occurred in a number of records. The disappearance of P waves in the human ECG (14, 15), and evidence of A-V dissociation in mammalian experiments (16) have been reported to result from

TABLE 1. EFFECT OF CaCl_2 UPON VENTRICULAR THRESHOLDS (TURTLE 1150 GRAMS)

TIME	THRESHOLD VOLTS
9:40-10:13	1.05-1.30
10:14-0.2 cc. of 10% CaCl_2 , intravenous	
10:15	1.30
:16	2.25
:17	2.25
:18	1.90
:19	1.90
:20	1.50
:21	1.50
:22	1.30

TABLE 2. EFFECTS OF CaCl_2 UPON THRESHOLDS OF VENTRICULAR MUSCLE IN TURTLE HEART DRIVEN AT CONSTANT RATE (TURTLE 925 GRAMS)

TIME	THRESHOLD VOLTS
12:52-1:00	3.0-3.5
1:02-0.3 cc. of 10% CaCl_2 , intra-sinus	
1:03	3.5
:04	4.1
:05	4.9
:06	4.6
:07	3.9
:08	4.1
:09-:11	3.9
:12	3.5
:13-:20	3.0

TABLE 3. EFFECTS OF CaCl_2 UPON AURICULAR THRESHOLDS IN VAGOTOMIZED-ATROPINIZED TURTLE (950 GRAMS)

TIME	THRESHOLD VOLTAGE ¹	TIME	THRESHOLD VOLTAGE ¹
12:50-12:55	3.0-3.4	:16-0.5 cc. of 1.5% CaCl_2 , intra-sinus	
12:57-0.5 cc. of 1.5% CaCl_2 , intra-sinus		:17	6.9
12:58	4.6	:18	6.1
:59	6.9	:19	5.4
1:00	7.5	:20	4.6
:01	6.1	:22	3.9
:02	4.6	:45	3.0
:05	4.6		
:10	3.9		
:15	4.6		

¹ Arbitrary units, not volts.

administration of calcium salts. Functional vagi were not necessary for the dissociation (16). Our results differ from those, perhaps only in degree. Bradycardia of vagal origin has been produced by small doses (16, 6, 5).

Excitability. The threshold changes produced by the administration of CaCl_2 , as measured by brief shocks, were variable. Significant increases in both auricular and ventricular thresholds were found in a large proportion of trials. No significant change was observed in a number of others with similar doses and conditions. Representative positive results are presented in tables 1, 2 and 3.

Table 1 shows the effect of an intravenous injection of 0.2 cc. of 10 per cent CaCl_2 . In this experiment the heart was beating at its own naturally initiated rate. In each trial, shocks were delivered during a period of three to five cardiac cycles at a frequency about three times the cardiac rate. Shocks were delivered therefore in all phases of the cardiac cycle. Two minutes after injection the threshold voltage had doubled. Four minutes after injection, it had passed over the maximum and was declining toward the control which was reached after about 8 minutes from the time of injection. The threshold remained stable within the control range (1.05–1.30 volts) during the next $3\frac{1}{2}$ hours, after which another injection was made. At this time a dose twice as large (0.4 cc. of 10 per cent CaCl_2) produced an approximately equal increase in threshold voltage. With the larger dose the duration of change was increased to about 10 minutes.

Table 2, from another experiment, also shows a rise of ventricular threshold upon intra-sinus administration of 0.3 cc. of 10 per cent CaCl_2 . In this experiment the heart was driven at a constant rate by shocks applied to the right atrium. The sino-atrial junction had been crushed. Testing shocks from the electronic stimulator were delivered at a constant moment in diastole. The control threshold is higher in this case, and the degree of rise of threshold, though larger in volts, is smaller in terms of percentage of the control.

The effects of CaCl_2 upon auricular threshold are shown in table 3. In this turtle the vagi had been sectioned and 1.2 mgm. atropine sulfate had been injected into the sinus to prevent changes in excitability that might result from responses to the electric shocks by vagus ganglion cells and terminal filaments. It was found that the absolute threshold of the auricular muscle was lower than that of the ventricle. The results are given in arbitrary voltage units, but not volts. The changes are similar in direction to those found in ventricular muscle.

It has been mentioned that in some trials CaCl_2 failed to produce a change in threshold. In the turtle used to produce the results in table 1, subsequent trials 1 hour and $2\frac{1}{2}$ hours after the first injection failed to produce significant changes in threshold, though the third dose (0.4 cc. of 10 per cent) was twice as large as the first. One hour after the third injection a repetition of the 0.4 cc. dose of 10 per cent CaCl_2 produced an increase in threshold approximately equal to that observed in the first trial (table 1). In the experiment that produced the results in table 2, a trial made upon the auricular muscle previous to the ventricular trial shown in the table failed to show a threshold change. Two subsequent trials upon the ventricle of this turtle also failed to show significant changes.

The inconsistencies in observed threshold changes cannot be accounted for as due to varying experimental conditions, although attention to detail is required. After injection of CaCl_2 solution into an abdominal vein, constriction of the vein often could be observed. The closure of the vein with resulting slow or incomplete passage of the solution to the heart probably accounts for some of the diminutions of effect before the venous constrictions were noted. However, this is not the sole cause of negative results. They were obtained in some trials after intra-sinus, intra-auricular, and intraventricular injections. A possible physiological reason for the variations is suggested in the discussion.

DISCUSSION

The differences in the time course of the several observed effects of calcium administration are of interest. With a moderately large dose such as 0.3 cc. of 10 per cent CaCl_2 the effect on contractility had a duration of about 2 minutes or less, the rise in threshold lasted about 8 to 15 minutes, and the sino-atrial dissociation which made its appearance after a delay of 8 to 15 minutes after injection persisted for about 3 hours. Another striking observation is the lack of cumulative effect of repeated injections of CaCl_2 upon contractility, excitability, A-V conduction, or intraventricular conduction. Cumulative effects were noted upon sinus rhythmicity, and possibly S-A conduction. The contractility response was repeatable over a period of several hours and after numerous injections. Even in the standstill and hypertonus after a large dose, when there was no circulation, the effect of excess calcium salt upon contractility was limited to a few minutes. It is obvious that in standstill calcium was not removed from the tissues. Therefore, recovery of responsiveness to the driving shocks applied to the right atrium, recovery of contractility of atria and ventricle, and the disappearance of hypertonicity all occurred while the concentration of calcium in the heart remained high. All observations agree in indicating that the presence of excess amounts of calcium has little or no effect upon excitability or contractility. The chemical state of the calcium is important. McLean and Hastings (17) have shown that only the ionized calcium affects contractility. Evidence from nerve studies has shown that the ionic calcium concentration in saline solutions with which the nerve is equilibrated (other cations remaining constant) governs the threshold (9, 7). It has been shown that the greater part of the calcium in CaCl_2 added to dog's blood rapidly becomes non-diffusible, and there is evidence for intracellular precipitation of added calcium (18). The rate of these changes from the ionized state probably governs the time course of the contractility and excitability effects.

The slow development and long persistence of S-A block may be interpreted as meaning that conduction through the S-A junctional tissues is depressed by a high concentration of total calcium within these cells.³

The finding that added calcium ions increased electrical thresholds in many trials and at the same time induced the discharge of ectopic impulses remains to be clarified. Certain observations from the literature can be correlated with these results to provide a hypothetical explanation. Rothberger and Winterberg (20) showed that excitation of the right or left accelerator nerves often caused ectopic beats in the right or left ventricle, but many trials gave negative results. Accelerator stimulation after suitable doses of CaCl_2 or BaCl_2 , when the salt alone was ineffective, regularly produced ventricular ectopic systoles. When the left accelerator was stimulated the ectopic foci were in the left ventricle and the right accelerator produced ectopic foci in the right ventricle. There were no negative trials, and it was evident that the calcium or barium salt potentiated the effects of accelerator stimulation. In sufficient dose, the salt produced ventricular arrhythmias without accelerator stimulation.

³ From experiments of a different kind, Means (19) has stated that the influence of calcium ion on the sinus rhythm depends on a different property of calcium from that which determines its relations to the mechanical response and to the propagated disturbance.

Lissak (21) demonstrated that excess calcium ions in Ringer's solution perfused through a frog's heart caused the liberation of sympathin without nerve stimulation. When the sympathetic supply to the heart was stimulated during perfusion the fluid acquired a greater sympathin effect.

From these findings it appears that calcium ions in suitable excess may liberate sympathin from sympathetic endings, and enhance its liberation by nerve impulses. This may possibly be adequate explanation for the ectopic discharges following the administration of CaCl_2 .

The majority of data on the threshold changes indicates that calcium ions have a threshold-raising effect on heart muscle similar to that found in nerve, which has been interpreted as increased membrane polarization. If this effect occurs in all heart muscle except in minute areas at sympathetic nerve endings, where the opposite effect may be produced by the liberation of sympathin, then the paradox ceases to exist. It seems possible that electrodes on the surface often might vary in their proximity to the sympathetic endings and that some of the threshold findings, which were inconsistent with the majority of results, may be due to closer proximity of electrodes to sympathetic endings in those trials.

In regard to the suggestion that the very local release of sympathin by calcium might account for ectopic discharges and threshold inconsistencies, questions concerning certain dissimilarities between the effects of calcium and of adrenalin have been raised. Further experimental evidence is needed before detailed discussion is justified.

SUMMARY

The changes produced by excess calcium chloride upon contractility, rhythmicity, sino-atrial conduction and excitability have been studied in turtle hearts *in situ*.

Small doses administered to turtles with hearts driven at a constant rate increased the height of contraction for a period of about one minute without an increase in tonus. Larger doses increased the height of the contraction summits to about the same extent as the small doses and considerably increased tonus (contraction remainder in diastole), diminishing the excursions. Very large doses quickly stopped the heart though the driving stimulator continued to deliver shocks of several times threshold intensity. The auricles and ventricles stopped in a contracted state. Early in calcium hypertonic standstill the degree of shortening varied between 35 and 75 per cent of that of a control contraction. This tonic shortening gradually relaxed during the few minutes of standstill. Rhythmic responses to the driving stimulator returned within 4 to 6 minutes. In hearts that were not driven, the reactions were similar. Automaticity was suppressed and hypertonic standstill supervened for a few minutes.

Electrocardiograms were recorded with leads arranged to include the sinus venous wave (designated as O). Suitable doses of CaCl_2 resulted in sino-atrial (O-P) dissociation which began after a latency of about 8 to 25 minutes and persisted for about 3 hours. A larger dose sometimes caused disappearance of the O wave (sinus standstill) for a brief period. During the sino-auricular dissociation there was some shifting of the atrial pacemaker which changed the P-R interval. Otherwise

P-R remained constant. Little change was seen in the shape or duration of the QRS complex. The Q-T interval changed only as the heart rate changed.

Threshold changes in a majority of trials consisted of a significant rise (reduced excitability) which passed through a maximum and returned to the control level within 7 to 15 minutes. The degree of rise for a given dose was variable and there were negative trials.

Ectopic auricular systoles were seen often during the period after the effect of calcium on contractility and tonus had passed the maximum and when the threshold to electric shocks was high.

In the discussion a hypothetical explanation for the excitability paradox (ectopic discharges during period of high threshold after calcium administration) is suggested.

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EFFECT OF ENERGY INTAKE ON HEART RATE IN HYPERTHYROIDISM INDUCED BY FEEDING THYROPROTEIN¹

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DURING the course of investigations to study the effect of thyroprotein (Protamone²) on milk production, longevity, reproduction and the circulatory system of dairy cows, it was noted that when these animals were fed according to body weight and milk production by the Morrison standards, heart rate was markedly increased by thyroprotein in the first part of lactation. This effect has been noted by other investigators (1, 2). However as lactation progressed and as the cows lost weight under the stimulation of thyroprotein, the heart rate decreased from levels of 90 to 100 beats per minute to about 60 beats per minute which is near the normal level for lactating cows. That this drop in heart rate was not altogether a result of the animals developing a tolerance to thyroprotein was indicated by the fact that the heart rate increased when extra feed was given. Body weight increased at the same time. These results suggested that thyroprotein might be less active when the amount of energy for metabolic purposes is deficient. On the other hand the decrease in heart rate could be a result of insufficient energy alone either from feed or body stores since Benedict and Ritzman (3) have shown that plane of nutrition affects heart rate. The present study was therefore undertaken to determine the interaction between energy intake and the action of thyroprotein on heart rate.

PROCEDURE

Two dairy steers, *A* and *B*, were fed thyroprotein at a rate of 1.5 grams per 100 pounds body weight during periods when their feed allotments were 50, 100 and 140 per cent of their calculated energy requirements. Each period lasted at least 30 days.

Another pair of steers, *C* and *D*, were given similar amounts of feed during the same periods without added thyroprotein and a fifth steer, *E*, was kept on a relatively constant intake over the whole experimental period to rule out possible seasonal effects.

Heart rates were obtained twice weekly in the morning before feed was given to the animals. The average heart rate for that portion of the period where maximum and constant effects of thyroprotein or feed intake were evident was used for comparison of the effects of the several procedures.

Received for publication January 9, 1948.

¹ This research was supported in part by an appropriation from Bankhead-Jones special research funds (Bankhead-Jones Act of June 29, 1935).

² Protamone, a product of Cerophyl Laboratories, Inc., Kansas City, Missouri, is a thyroactive iodinated casein containing thyroxine.

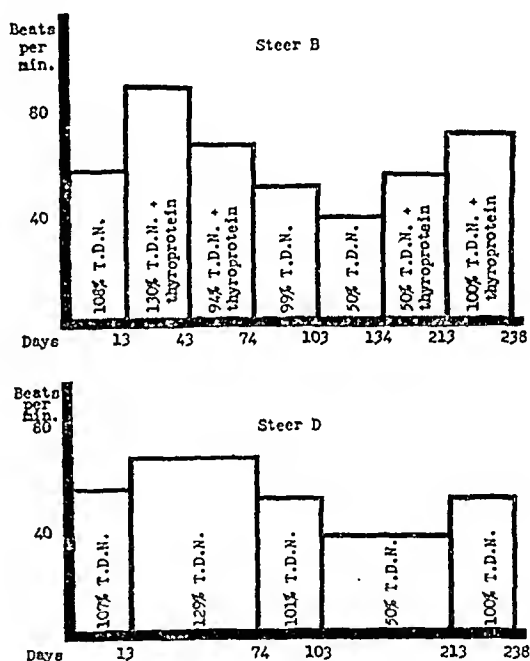
Feed and thyroprotein were adjusted at 10-day intervals on the basis of body weight obtained at similar intervals. Changes in energy intake were in most instances made by increasing or decreasing the grain portion of the ration.

RESULTS

The consumption of feed and variations in procedure and the heart rate under the various conditions for one steer of each pair are shown in figure 1.

An increase of feed in addition to thyroprotein from the 13th to the 43rd day (steer B) increased the heart rate from 59 to 91 beats per minute. When the feed was reduced during the next period the heart rate dropped to 70 beats per minute, a change of 21 beats. Of the change in rate produced in the period from 13 to 43 days the extra feed therefore accounted for an increase of 21. The thyroprotein added at

Fig. 1. EFFECT OF THYROPROTEIN and energy intake on the heart rate of steers. The % T.D.N. refers to the actual consumption of total digestible nutrients based on the calculated requirements of each individual.



the same time was responsible for the additional increase of 11 beats per minute. The difference in heart rate in the period 74 to 103 days as compared to the previous period is due to the withdrawal of the thyroprotein, and the thyroprotein effect from 43 to 74 days was therefore 16 beats per minute. In this manner the effect of thyroprotein at the various levels of energy intake and the effect of various energy intakes on heart rate has been determined. The effect of increasing or decreasing energy consumption on heart rate uncomplicated by administration of thyroprotein for steer D is also shown in figure 1.

Marked changes in heart rate occurred as a result of alterations in energy consumption or as a result of feeding thyroprotein at different levels of energy intake. The complete data for all four steers and the control are shown in table 1.

It will be noted that as feed consumption increased, heart rate also increased and this general relationship was true whether thyroprotein was fed or not. The heart rates at all levels of consumption were higher when thyroprotein was fed but, in spite

of the hyperthyroidism induced by the thyroprotein which was severe enough to cause loss of body weight even when the animals were being fed in excess of requirements, increases or decreases of feed consumption resulted in changes in heart rate.

Booth, Elvehjem and Hart (3) have presented results which are an apparent contradiction of this general relationship. They state: "Little if any correlation between heart rates and the level of concentrate feeding was apparent, contrary to the results of Moore" (5). That they came to such a conclusion is not surprising but their results and those obtained by Moore (5) cannot really be compared. The Wisconsin workers did not have accurate feed consumption data since they fed hay *ad libitum* and approximately 15 pounds of silage and only had accurate records on the concentrates fed. In addition some of their animals were on pasture in one trial. In the present work and in that reported by Moore accurate records were kept on the total feed consumption. On this basis there is a relationship between heart rate and

TABLE 1. HEART RATE (BEATS PER MINUTE) AT VARIOUS LEVELS OF T.D.N. INTAKE WITH AND WITHOUT THYROPROTEIN

FEED INTAKE	STEER A		STEER B		STEER C	STEER D	STEER E ¹
	1	2	1	2	1	2	
50-52% of requirements	40	54	42	58	45	40	59
89-108% of requirements	67	80	59	70	56	58	66
	56	67	54	74	65	55	59
					63	55	61
115-131% of requirements	—	90	—	91	70	70	65

1. Without thyroprotein. 2. With thyroprotein.

¹ Constant feed intake throughout.

feed consumption and while adjustments in feed intake were usually made by increasing or decreasing the concentrate portion of the ration, a correlation between heart rate and concentrate feeding could only be expected if the remaining parts of the ration remained constant, a condition which would almost certainly not hold where hay was fed *ad libitum*. In addition, we have observed variation in heart rate between cows at similar feed levels and stages of lactation and as a result of variations in the technique of determining rates (unpublished data). The initial heart rates of some of the cows used by Booth *et al.* seem to be above normal and may have been due to some of these factors. These high initial rates would make a correlation between concentrate feeding and heart rate still less probable.

The effects of changes in energy intake are shown in table 2. In this table the alteration in heart rate produced by addition or withdrawal of feed have been reduced by calculation to a common basis of a 10 per cent increase or decrease in feed consumption. This was necessary because of the variation in feed intake of the various steers. The left-hand column indicates the amount of feed being consumed when additions or decreases in feed were instituted.

If the one very high value of 11.1 is excluded from the data, a 10 per cent change in feed consumption produced on the average a change of heart rate of 3.6 beats per

TABLE 2. CALCULATED CHANGES IN HEART RATE INDUCED BY CHANGING FEED INTAKE

T.D.N. INTAKE WHEN FEED CHANGES MADE	CHANGE IN HEART RATE (BEATS/MIN.) PRODUCED BY 10% INCREASE OR DECREASE IN T.D.N.			
	Steer A	Steer B	Steer C	Steer D
50% of requirements	2.6 ¹	3.2 ¹	3.2	3.0
94-101% of requirements	3.3	2.5	11.1 4.6	5.5 3.0
115-131% of requirements	2.5 ¹	5.7 ¹	2.2	5.3

¹ Thyroprotein being fed when these determinations were made.

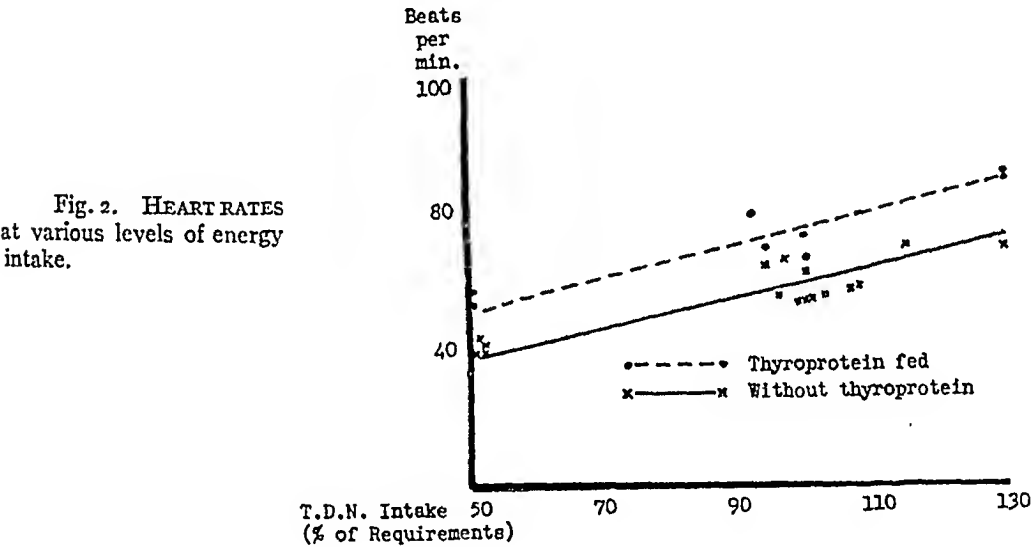


TABLE 3. INCREASE IN HEART RATE INDUCED BY FEEDING THYROPROTEIN AT DIFFERENT LEVELS OF FEED INTAKE

FEED INTAKE	INCREASE IN HEART RATE (BEATS/MIN.)	
	Steer A	Steer B
50% of requirements	14	16
94 and 96% of requirements	24	16
131% of requirements	13	11

minute. The change in rate was relatively constant irrespective of the level of energy intake at the time that changes in intake occurred. Similarly the presence of thyroprotein in the ration did not affect the relative values obtained. Whether or not this same relationship would hold in still more severe instances of hyperthyroidism as might be encountered in the human subject has not been determined.

The effect of thyroprotein on heart rate was also relatively constant over the whole range of energy intake which was studied. As noted previously (table 1), thyroprotein increased heart rate at all levels of energy intake as compared to rates observed at similar levels of intake without thyroprotein. The two sets of values parallel one another, as is shown in figure 2, over the range of intake which was studied. This relationship is even more striking when the variation in energy intake of individual steers which existed at certain levels of consumption is taken into consideration.

The observed and calculated changes in heart rate produced by feeding thyroprotein are shown in table 3. With the exception of one high value, the increases in heart rate were not significantly different at the various levels of feed intake. These data indicate that the amount of feed being consumed did not alter the essential activity of thyroprotein as far as its effect on heart rate was concerned.

SUMMARY

The heart rate of hyperthyroid steers can be materially altered by varying feed intake. Increases or decreases of energy consumption did not change the essential activity of thyroprotein on heart rate. The effects of thyroprotein and energy seem to be additive.

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THIAMINE DEFICIENCY IN NORMAL RATS AND IN RATS MADE DIABETIC WITH ALLOXAN¹

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IN VITRO studies have established that thiamine is an essential factor in the oxidation of certain intermediary products of carbohydrate metabolism. However, *in vivo* observations usually fail to show any definite alteration in carbohydrate levels in the blood until a condition of severe deficiency is reached. While Monauni (1) reported the occurrence of a hypoglycemia and Drummond *et al.* (2) a hyperglycemia in polyneuritic animals, it is generally agreed that animals with a mild to severe polyneuritis have decreased tolerances for carbohydrate (3-5). In addition to a decreased tolerance, Abderhalden *et al.* (6) and Vorhaus *et al.* (7) found increased liver glycogen levels. Styron *et al.* (8) observed no impairment of carbohydrate tolerance in normal and diabetic animals until there was a marked thiamine deficiency. Smith and Mason (9) gave two human diabetics thiamine-deficient diets and although a deficiency state was not reached, the diets had no influence on the intensity of the diabetes.

Since the relation of thiamine deficiency to diabetes has received only limited attention, the present study was undertaken to obtain further information on the problem. Normal animals were studied along with the diabetics so that the two groups could be compared. This investigation is somewhat similar to that of Styron *et al.* (8), but differs principally in that the rats here used were made diabetic with alloxan rather than by pancreatectomy and were studied under pair-fed conditions.

EXPERIMENTAL PROCEDURE

Thirty normal and 30 diabetic adult male rats of the Long-Evans strain were divided into a control group of 10 normal and 10 diabetic animals and an experimental group of 20 normal and 20 diabetic. The diabetes had been produced by subcutaneous or intraperitoneal injection of 125 mgm. of alloxan per kilogram of body weight. For the diabetic study only such rats were chosen as were severely and permanently diabetic, as shown by 3+ and 4+ urine sugars over a preceding period of 1 or 2 months.

The rats were fed an adequate basal diet (table 1) for 2 or 3 weeks before being paired. The normal rats were paired with each other according to similarity in body weight and food consumption; the diabetics were paired, as well as possible, according to similarity in urine volume and urine and blood sugars as well as in body weight and food consumption. One animal of each pair received the thiamine-deficient diet (basal diet without added thiamine), while the other animal was continued on the basal diet but was limited to the same amount of food as was eaten by its pair-mate. When anorexia caused a reduction in food intake of the rats on the deficient diet, the pair-mates received half of their food allowance in the morning and the other half in the late afternoon to avoid long fasting periods and the possibility of ketosis.

Received for publication June 12, 1948.

¹ Supported by a grant from Hoffmann-La Roche, Inc.

The urine of the diabetic animals was tested qualitatively for sugar and acetone bodies every two days. Quantitative blood and urine sugar determinations were made weekly, on nonfasted rats, by the method of Somogyi (10).

Both rats of a pair were killed when either the thiamine-deficient animal reached a state of severe polyneuritis or the pair-mate, because of the semistarvation diet, became moribund. The 10 normal and 10 diabetic control rats had body weights similar to the maximum weight observed in individuals of each thiamine-deficient group before anorexia became evident. In addition, the severity of the diabetes in the control diabetic rats was similar to that found in the diabetic animals on the deficient diet. The left kidney, adrenals, thyroids and pituitary were removed from all animals and were used for weight comparisons and tissue studies.

TABLE I. COMPOSITION OF BASAL DIET

CONSTITUENTS	AMOUNT gm.	CONSTITUENTS	AMOUNT mgm.
Casein (vitamin-free) Labco	18.0	Thiamine	0.5
Sucrose	30.0	Niacin	1.0
Starch	39.0	Pyridoxine	0.5
Crisco	5.0	Ca-pantothenate	1.5
Wesson oil	5.0	Riboflavin	0.5
Salt mixture (Hubbell, Mendel & Wakeman)	3.0	Haliver oil	0.2
Choline	0.1		

RESULTS

Physiological Effects

The rats remained in excellent physical condition until the polyneuritis developed in the thiamine-deficient animals or their pair-mates became moribund. Even though the diabetic rats had nonfasting blood sugar levels ranging from 600 to 1000 mgm. per cent, they did not exhibit ketosis at any time.

The food intake of the series of normal rats is not presented because it followed a very similar pattern in each deficient animal. During the control period the normal rats ate on an average of 15 to 18 grams of the basal diet per day. Ten days following the first signs of anorexia caused by the thiamine-deficient diet these rats ate an average of 3 to 7 grams of food per day; near the end of the deprivation period they ate from 2 to 5 grams. Animals which failed to eat on certain days are not included in the averages. The food consumption of the diabetic animals was greater as a result of the disease. The daily intake of the paired groups was generally between 20 and 40 grams (data of two typical pairs of diabetics are shown in figure 2). Soon after the thiamine-deficient diet was introduced there was a marked reduction in food intake of the diabetic animals but the rate of reduction was more variable than that found in the normal thiamine-deficient rats. Near the termination of the experiment the food intake of the diabetic-deficient rats was usually a little higher than in the normal deficient rats.

Following the introduction of the deficient diet the normal rats showed a loss of appetite after an average of 15.4 days (range +19 and -10) and polyneuritis after an average of 48.2 days (range +53 and -37). The diabetic rats, on the other hand, showed the loss of appetite after an average of 12.8 days (range +16 and -9) and polyneuritis after an average of 41.6 days (range +69 and -29). In both

normal and diabetic rats, 3 pair-fed animals of each group became moribund because of the semistarvation diet just before or at the same time as the polyneuritis became evident in the others.

The great variation seen in the time that polyneuritis appeared in the thiamine-deficient rats might be interpreted as indirect evidence that some of the rats were synthesizing thiamine. It has been shown that rats, under certain conditions, can synthesize vitamin B₁ (11). Furthermore, since 30 per cent of the pair-mates were moribund, the question arises as to the cause of death in the polyneuritic animals.

THIAMINE DEFICIENCY IN NORMAL RATS

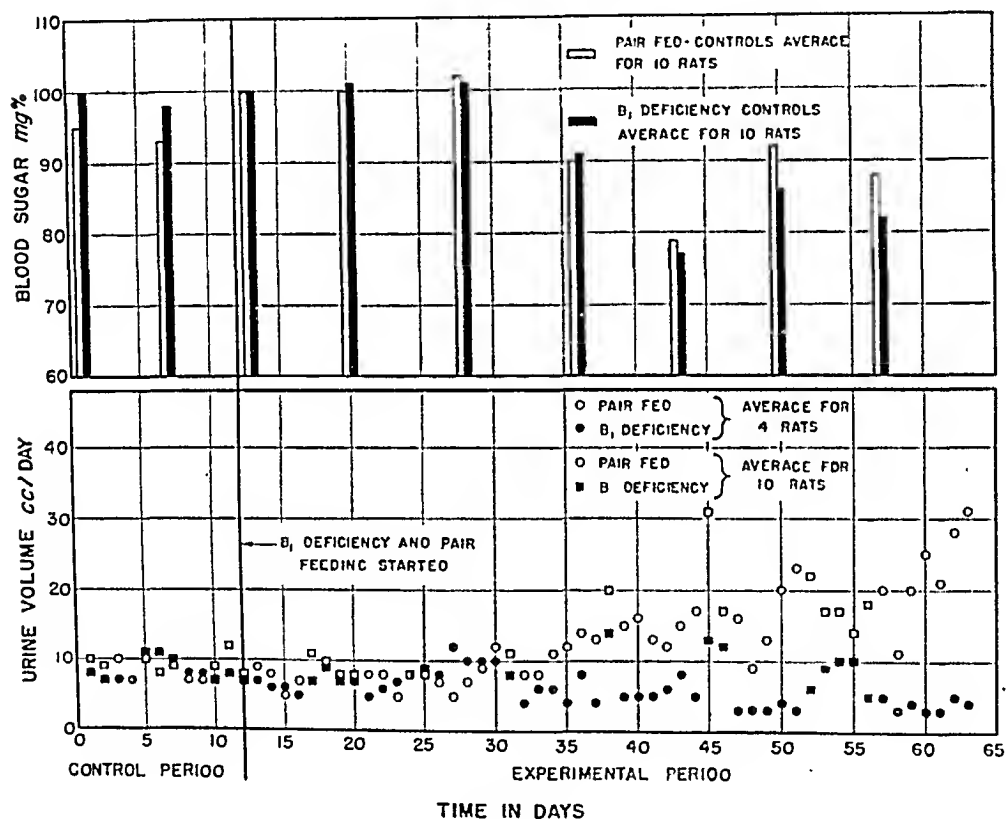


Fig. 1

Inanition may be the primary cause and the polyneuritis becomes more discernible, or at least appears more severe, because of the cachexia associated with the reduced food intake.

Average blood sugar values for the normal rats during the control period were a little lower in the animals which were to be pair-fed (fig. 1). Beginning with the thiamine-deficient period, the blood sugar values were similar during the first three sampling days and then decreased. However, as they decreased the average values became slightly higher in the normal pair-fed rats. The maximum and minimum values were within the range found in normal rats in absorptive and postabsorptive states. Considerable variation was seen in individual blood sugar values for each

pair of the diabetic rats. This was particularly evident in rats 35 and 36 (fig. 2). The blood sugar levels of the diabetic rats declined, as would be expected, with the reduced food intake. The average values during the control period for the 12 diabetic rats which were to receive the deficient diet was 761 mgm. per cent and for the animals which were to be pair-fed was 813 mgm. per cent, while at the termination of the experiment these values had decreased to 309 and 389 mgm. per cent, respectively.

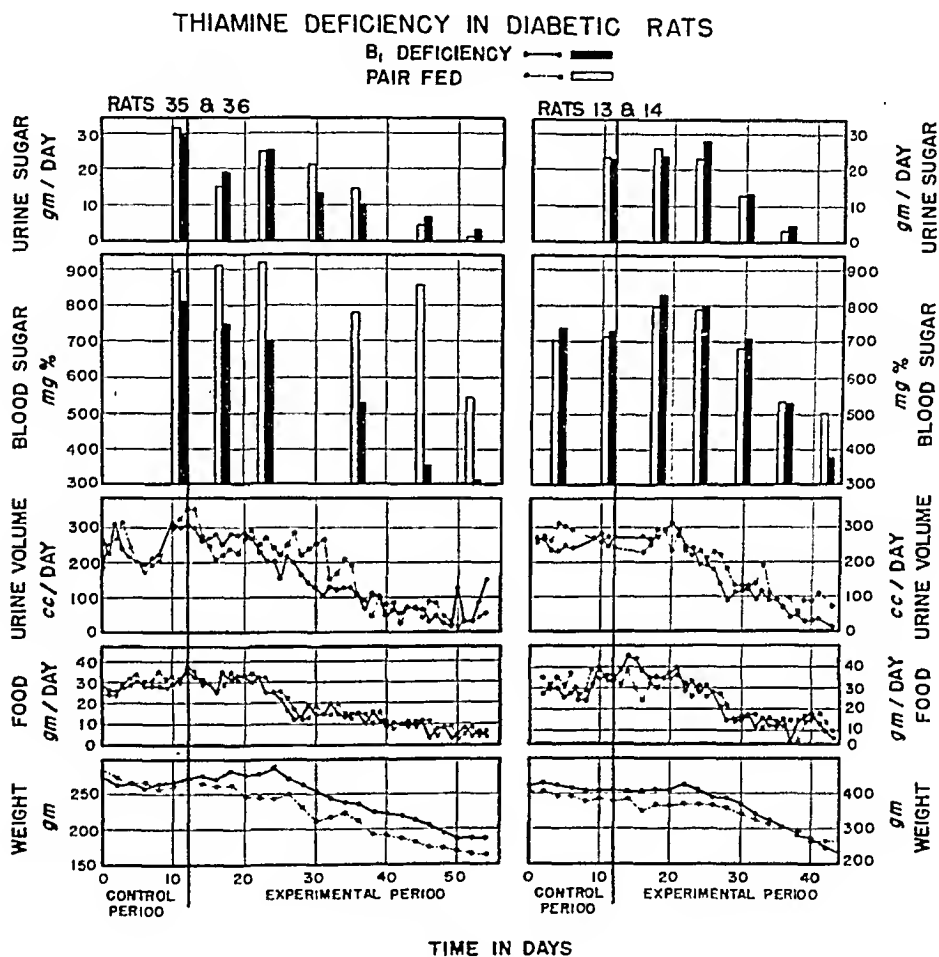


Fig. 2

In the normal series of rats urinary sugar was excreted in negligible amounts. In the diabetic animals, however, sugar excretion generally followed a pattern similar to that for food intake. When food consumption was high there was marked glycosuria, but as food intake declined, sugar excretion was correspondingly reduced (fig. 2). There was usually no marked difference in the excretion rate of the deficient and pair-fed animals.

The animals which received the thiamine-deficient diet showed the usual loss in body weight. The weight loss was not as marked in the pair-fed animals of each group (table 2), but the average initial maximum weight was a little less in these

TABLE 2. EFFECT OF THIAMINE DEFICIENCY ON BODY AND ORGAN WEIGHTS
(AVERAGES FOR 10 RATS IN EACH GROUP)

CONTROL	BODY WT.			ORGAN WT.		
	gm.			mgm.		
Normal	43 ¹					
Rt. kidney				1549.0±81.0 ²		
Adrenals				41.6± 2.2		
Thyroids				18.2± 1.7		
Pituitary				12.4± 0.3		
Diabetic	369					
Rt. kidney				2053.0±121.0		
Adrenals				52.4± 2.8		
Thyroids				17.4± 1.2		
Pituitary				11.6± 0.4		
EXPERIMENTAL	BODY WEIGHT			ORGAN WT.	MG/100 GM. BODY WT.	DIFF. IN ORGAN WT., EXP. & CONTROL GROUPS
	Max. wt. in exp. period	Terminal wt.	Wt. Loss			
	gm.	gm.	%	mgm.		%
Normal thiamine-deficient	429	217	50			
Rt. kidney				1244.0±51.0 ²	575±9.0 ²	-20
Adrenals				50.9± 3.8	23±1.0	+18
Thyroids				15.7± 1.0	7±0.7	-14
Pituitary				8.5± 0.3	4±0.2	-31
Pair-fed rats for normal thiamine-deficient group	415	242	42			
Rt. kidney				912.0±45.0	383±9.0	-41
Adrenals				42.8± 2.0	18±0.9	+ 3
Thyroids				15.3± 0.8	6±0.3	-16
Pituitary				9.5± 0.7	4±0.3	-23
Diabetic thiamine-deficient	371	234	37			
Rt. kidney				1353.0±90.0	599±48.0	-34
Adrenals				54.2± 2.8	24± 1.8	+ 3
Thyroids				18.9± 1.4	8± 0.5	+ 8
Pituitary				8.3± 0.4	4± 0.3	-29
Pair-fed rats for diabetic thiamine-deficient group	354	232	34			
Rt. kidney				1105.0±90.0	487±31.0	-46
Adrenals				49.3± 2.1	22± 1.6	- 6
Thyroids				15.6± 1.0	7± 0.5	-10
Pituitary				9.8± 0.7	4± 0.2	-16

¹ From animals having maximum weights observed in deficient rats before anorexia became evident. The severity of the diabetes in the control diabetic animals was similar to that observed in the experimental animals.

² Standard error.

rats. Regardless of the initial weights, the average terminal weights of the different experimental groups were fairly similar.

The thiamine-deficient diet appears to have a greater influence in altering appetite than in altering blood sugar levels, since carbohydrate levels are very similar, before polyneuritis becomes evident, in both the deficient animals and their pair-mates. Perhaps the small amount of thiamine which is present in the deficient diets or the thiamine which may be synthesized is sufficient for the part it plays in the oxidation of carbohydrates but not sufficient to maintain the appetite of the rats or sufficient to protect the nervous system, for various periods of time, from the characteristic neuritis.

It is true that near the termination of the experiment the deficient rats showed a tendency towards lower blood sugar levels and a greater weight loss than their pair-mates. The reason for this is not evident from these studies but it may result at this time from a decreased utilization of carbohydrate or a decreased absorption of food from the intestines. Such an alteration in the absorptive rate has been described by Free and Leonards (12).

The urine volumes of the normal pair-fed animals displayed an unexpected pattern. Although exhibiting some variation, the average urine volumes for the normal rats receiving the deficient diet were fairly similar throughout the experiment. However, about the time the food intake of their pair-mates was reduced, the urine volumes of these animals increased and became progressively elevated until the termination of the experiment (fig. 1). The urine output of the deficient and pair-fed diabetic rats did not exhibit such a marked difference. Because of the diabetes, the urine volumes of these rats were very high initially but declined in both groups with the reduced food intake. However, with limited rations the pair-fed diabetic animals showed a tendency to excrete more urine than the deficient rats (fig. 2). Michelson (13) has found increased urinary excretion in human beings who were given a semistarvation diet but the exact cause was not understood. Perhaps when the amount of food is limited the animals drink more water to create a feeling of fullness.

Organ Studies

Weight Changes. There was considerable variation in the organ weights of the normal and diabetic controls as well as of the thiamine-deficient rats (table 2). The kidneys and adrenals from the diabetic animals were larger than those in the normal rats but the thyroids and pituitaries of the two groups were about the same size. The organ weights of the experimental animals exhibited less departure from the controls, when considered as percentage change, than did values for total body weight.

The kidneys showed loss in weight in each of the thiamine-deficient groups when compared with the controls, but with the exception of one pair of diabetic rats, this loss was less than in the pair-fed rats. The weight decrease in the kidneys of the pair-fed animals was comparable to the loss in body weight. The terminal weights, calculated as mgm./100 grams body weight, were smaller in the pair-fed animals than in the deficient rats. The weights of the adrenals in the deficient

animals did not vary as much from control values as the kidneys. Instead of showing a marked loss in weight, the adrenals maintained their weight and were actually hypertrophied in the normal thiamine-deficient rats. The adrenals of the deficient rats were a little larger than those from the pair-fed. The thyroids of the deficient rats were slightly but not significantly larger than those from pair-fed controls.

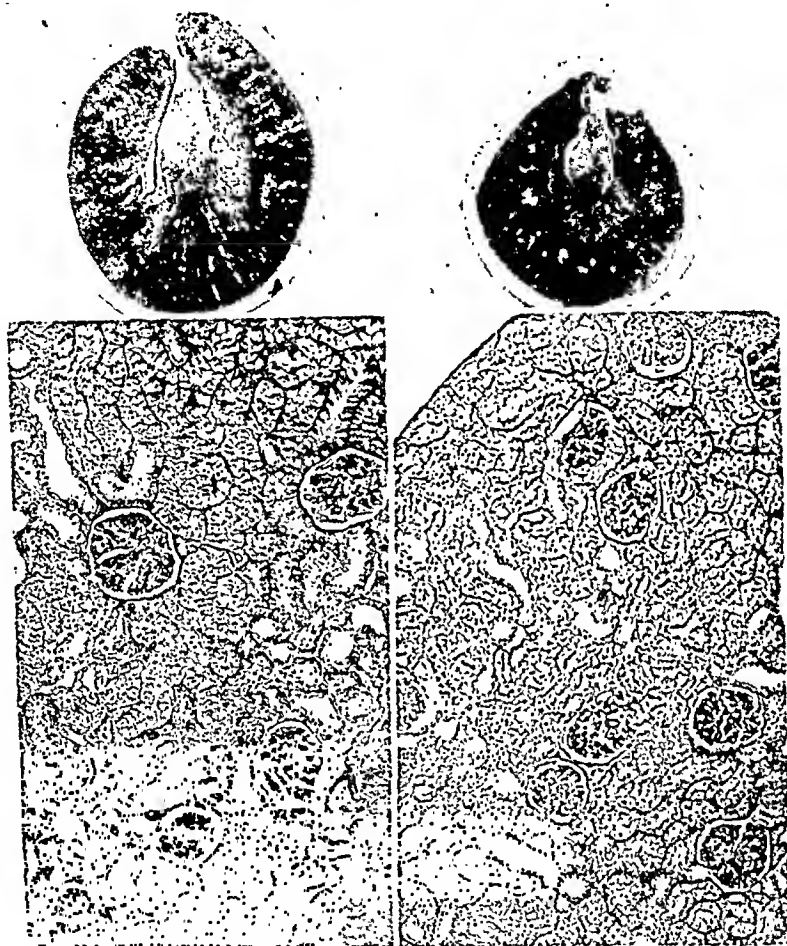


Fig. 3. EFFECT OF B_1 DEFICIENCY ON kidney size and structure. *Upper left*: section of kidney from B_1 -deficient diabetic rat. $\times 3$. Body wt., 180 gm.; kidney wt., 1336 mgm. *Upper right*: section of kidney from pair-fed control diabetic rat. $\times 3$. Body wt., 174 gm.; kidney wt., 722 mgm. *Lower*: sections of cortex of above kidneys, $\times 70$. Note larger Malpighian bodies in kidney of thiamine-deficient rat (*left*).

When compared with the controls the pituitaries of the experimental animals showed a loss in weight which was less than, but proportional to, the loss in body weight. When the pituitary weights were calculated as mgm./100 gram body weight, values were identical for each of the experimental groups.

Histological Changes. Several organs were studied histologically in an effort to determine if the thiamine deficiency had produced any alteration in organ structure. Organs of the deficient animals were compared with their pair-mates as well as with the organs of their respective normal and diabetic controls. The kidneys were the only organs which exhibited histological modifications which could be

attributed solely to thiamine deficiency. In 19 out of 20 pairs of normal and diabetic experimental rats, the kidneys were larger in the deficient animals than in their pair-mates. Furthermore, the Malpighian corpuscles were larger in the deficient animals and roughly proportional to the size of the kidneys (fig. 3). The kidney from one deficient diabetic rat was smaller than the kidney from its pair-mate, but the average diameter of Bowman's capsules was a little larger in the kidney from the deficient animal. In addition to the larger Malpighian corpuscles in the deficient rats, the kidney tubules appeared larger and the blood vessels more congested, particularly in the diabetic animals. All the kidneys from diabetic animals showed the usual changes associated with severe alloxan diabetes.

McCarrison (14) reported that vitamin B-deficient diets caused an atrophy of the kidneys, and Martinez (15) found edema in the glomerular zone and tubular atrophy in thiamine-deficient animals. In the present study, these pathological changes were not found.

SUMMARY

Normal rats and rats made diabetic with alloxan were given a diet deficient in thiamine and were studied under paired-feeding conditions. The thiamine-deficient diet appeared to have a greater influence in altering appetite than in altering blood sugar levels, since the latter were very similar, before polyneuritis became evident, in both deficient and pair-fed animals. Near the termination of the experiment, the deficient rats showed a tendency towards lower blood sugar levels and a greater weight loss. This may have resulted from a decreased utilization of carbohydrate or decreased absorption of food from the intestines.

The kidneys of thiamine-deficient rats were larger than those of their pair-mates and had larger Malpighian bodies. The urine volumes, however, were larger in the pair-fed rats than in the deficient animals, particularly in the normal series.

The primary cause of death in the deficient rats was probably not polyneuritis, per se, but inanition.

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TRYPTOPHANE AND BLOOD SUGAR LEVELS

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THAT dl-tryptophane may lower blood sugar levels was first suggested by Crowell and Turner (1). This was reported in letter form and was mentioned again by the same authors in a later paper (2). In their experiments a group of 9 young adults were given dl-tryptophane in doses varying from 0.14 to 0.32 grams adjusted to body weight for eight days, with a one-day rest on the fifth day. The material was taken in powder form since previous ingestion in gelatin capsules had proved less effective in changing salivary amylases, which was their immediate objective. They report blood sugar levels before tryptophane and two hours after the last dose of the amino acid. Among their 9 subjects, only 1 failed to show a decrease in the blood sugar levels. They found an average drop of 26 mgm. per cent in the other 8 cases, the lowering ranging from 8 to 50 mgm. per cent. Because of the possible therapeutic implication as well as the little known mechanism of activity of this amino acid, further study of the problem seemed warranted.

PROCEDURE

Normal male students at this university were used as subjects, with ages ranging from 19 to 31. Weight variations are mentioned in the tables below. Daily doses of dl-tryptophane¹ were fed to these students for a period of two weeks without interruption.

No dietary restrictions were imposed upon the subjects. The material was given in gelatin capsules because of ease of administration since we could see no reason why the gelatin should interfere with the overall picture to be obtained. Blood sugars were drawn in all cases after nine hours of fasting. The initial dose of tryptophane was taken immediately following the first blood sugar. Sample sugars were taken throughout the two-week course and the final level obtained 24 hours after the last dose of tryptophane. In a few cases another blood was examined one week later to check for possible latent effects. All blood sugars were determined by the Folin-Wu method using the Klett-Summerson photoelectric colorimeter. The students were divided into various groups, but in several cases after a two-week rest were used in the next higher dosage group.

RESULTS

Table 1 shows the mean values of blood sugars of the subjects taking dl-tryptophane over a two-week course.

Received for publication May 7, 1948.

¹ The dl-tryptophane was kindly furnished by Merck & Co., Inc., Rahway, N. J.

Column A gives the data for the 0.25 gram per day group, and no significant change can be noted. With a total of 93 blood sugar determinations in 14 subjects in this group only 2 showed significant changes at the end of the experiment. Of these, 1 was not reliably reported on the last day of the experiment, and some error in the analysis suspected. This was supported by a return to his original level in subsequent follow-up studies. The other individual who showed a change failed to do so when placed on higher dosages of the amino acid. Although marked fluctuation occurred in the series, with the largest individual range from 84.1 to 71.3 mgm. per cent, we could not establish a definite trend for either an increase or a decrease on any 1 day.

TABLE 1

TIME	A	B	C	TIME	A	B	C
days				days			
0	86.6	79.8	89.7	9	85.7	91.0	89.8
1			90.3	11	85.0		
2	89.4	86.4	90.6	12	82.6		
4		85.5		14	86.6	88.9	
7	85.1	84.8	93.0	21	84.3	89.0	

Mean values, blood sugars (mgm. per cent), following ingestion of dl-tryptophane. *Column A*: results of 14 cases (wt. 150-200 lbs.) on 0.25 grams daily. *Column B*: results of 11 cases (wt. 110-170 lbs.) on 0.50 grams daily. *Column C*: results of 3 cases (wt. 155-190 lbs.) on 1.0 grams daily.

TABLE 2

TIME	CONTROL	0.25	0.50	1.0
hr.				
0	99.9	90.1	88.1	95.9
1	97.4	92.5	90.1	95.6
2	98.7	88.2	88.2	95.9
3	101.4	89.5	89.2	94.2

Mean values, blood sugars (mgm. per cent), after ingestion of dl-tryptophane in 0.25, 0.50, and 1.0 gram capsules with controls for 4 cases over a 3-hour period.

Column B gives the results of the feeding of 0.5 gram of tryptophane per day. Of the 11 cases cited here, 5 showed significant changes, all increases, ranging from 10.0 to 28.4 mgm. per cent. This in part accounts for the low mean value of the blood sugar at the start of the experiment, for the other 6 subjects showed differences in their initial and final blood sugars of only -1.6 to 7.2 mgm. per cent. In only 1 case was a possible error in determination suspected, that being an initial reading of a subject who in every subsequent test showed a uniformly higher value. We feel reasonably certain that his initial value should have been higher and that his total gain of 28.4 mgm. should be less. It is interesting to note that in no case was a marked decrease encountered.

In *column C* are shown results of the ingestion of 1.0 gram of tryptophane daily.

Since no significant change was obtained in the 3 cases on 1 gram daily, the possibility remained to be investigated that the Crowell-Turner group had picked up a transient change in the blood sugar since their last blood was drawn two hours after the last dose of tryptophane. To investigate this we drew a fasting blood sugar, and immediately after this the subject ingested his tryptophane. Then at varying times afterwards bloods were drawn and examined. Table 2 summarizes these experiments.

If we contrast the hourly fluctuations in the blood sugars with no tryptophane with those given the amino acid, we find no significant changes.

DISCUSSION

That tryptophane should have any permanent effect on blood sugar levels seemed to us rather remote. Tryptophane is not present in insulin itself (3) and is not converted into glucose. That it might be present in some remote control mechanism or might exert some effect upon the liver remained as possibilities. A possible mechanism for its action in lowering blood sugars was suggested by Crowell and Turner (2). We, however, have not been able to demonstrate either a sustained significant increase or decrease within our experimental conditions, or a transient effect.

SUMMARY

The effect of dl-tryptophane in blood sugar levels has been studied using normal males as subjects. Doses of 0.25, 0.50, and 1.0 gram per day for 14 days failed to lower the blood sugar levels. In 5 of the 11 cases given 0.5 gram per day, a significant increase was noted. An attempt was made to pick up a transient effect of tryptophane on blood sugar levels some 1 to 3 hours after ingestion, but no effect was found.

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FLAME PHOTOMETER DETERMINATIONS OF SODIUM AND POTASSIUM IN RELATION TO POSSIBLE INTERFERENCE BY PHOSPHATE¹

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CRISMON (1) has reported that analyses of tissue ash of muscle for sodium and potassium by the flame photometer and by chemical methods show agreement only after phosphate has been removed (by precipitation with CaO) in the aliquots analyzed by the flame photometer. Thus, in the presence of phosphate, sodium readings are reported to be 24 to 53 per cent lower by flame photometry and potassium readings were found to be 19 to 29 per cent lower than those determined by chemical procedures. Standard solutions of sodium phosphates were also reported to give only about 50 per cent of the values for sodium by flame photometry compared to values after phosphate removal. Crismon suggests that determinations of urinary sodium and potassium with the flame photometer should be checked for effect of phosphates.

Because we have made extensive use of the flame photometer in determining sodium and potassium in urine and in brain and muscle (2, 3), the present series of experiments was undertaken to determine the extent of interference caused by phosphates.

URINES

A total of 39 urines from 13 men were analyzed for sodium and potassium using the Perkin-Elmer Flame Photometer (Model 18). One series of aliquots of these samples was analyzed directly for sodium and potassium while a second series of aliquots was treated with CaO to remove phosphates.

A. First Series of 18 Urines. The series of aliquots which contained unremoved phosphates were prepared by pipetting a 1 cc. sample of urine into a 100 cc. volumetric flask and making solutions up to volume with distilled water. Direct readings for sodium on the flame photometer of these 1/100 dilutions of the urine samples were then made. For potassium 1/300 dilutions were used. Our flame photometer

Received for publication June 11, 1948.

¹ This work was aided by Contract N6 ori-197 of the Office of Naval Research.

is regularly calibrated so that 19.9 parts per million (ppm.) of potassium and 50.0 ppm. of sodium read 100 on the dial calibrated from zero to 100.

Phosphate-extracted series. The series of aliquots which were designed to be free of phosphates were prepared by pipetting a 1 cc. sample of urine into a 10 cc. volumetric flask and making solutions up to volume with distilled water; CaO (C.P.) was added until no further precipitate was formed and solutions were subsequently centrifuged and filtered through ash-free filter paper (S and S No. 589 Blue Ribbon). Control solutions of NaCl and KCl were concurrently filtered in order to rule out possibilities of contamination by the filter paper. We have found that neither centrifugation nor filtration of the urines varied sodium or potassium values. The 10 cc. solutions were then made up to 100 cc. and analyzed directly on the flame photometer for sodium at 1/100 dilution, while potassium readings usually necessitated a dilution to 1/300 to bring the values onto our calibration curve for potassium.

The analyses of three urine samples for each of 6 men gave mean values for sodium excretion of 162 mgm. per hour before removal of phosphate and 157 mgm.

TABLE 1. pH, PHOSPHATE, SODIUM AND POTASSIUM VALUES BEFORE AND AFTER CaO TREATMENT TO REMOVE PHOSPHATE (MEAN OF 21 SAMPLES)

pH		PO ₄ mgm. %		K EXCRETED mgm./hr.		Na EXCRETED mgm./hr.		% PO ₄ REMOVED
pre CaO	post CaO	pre CaO	post CaO	pre CaO	post CaO	pre CaO	post CaO	after CaO
6.7	10.7	1.14	0.11	192	195	258	279	90.4

per hour after phosphate removal from aliquots of the same samples. These same 18 samples gave mean values of potassium of 193 mgm. per hour before phosphate removal and 208 mgm. per hour afterwards. Application of Fisher's *t* test shows these means not to be significantly different. In the case of both sodium and potassium the mean values of the phosphate-containing and the phosphate-free samples do not show statistically significant differences.

B. Second Series of 21 Urines. Using the same procedures that have been outlined above, three samples of urine collected from each of 7 men were analyzed by the flame photometer. In the case of these urines pH and phosphate values after the method of Fiske and Subbarow were obtained on all of the aliquots. Table 1 shows the pH, phosphate content and sodium and potassium values both before and after CaO treatment, as well as the percentage of phosphate removed from solution. As in the case of the first series of urines, the mean values of sodium and potassium excreted (mgm./hr.) as determined by the flame photometer in the presence of phosphates do not show significant differences from values obtained after phosphates have been removed.

Averaging the values before and after phosphate removal for all 39 urine samples and expressing the results in mgm./hr. excreted, we find a sodium value of 210 before and 218 after phosphate removal and a potassium value of 192 before and 201 after phosphate removal. The respective means thus agree to within 4 per cent.

TISSUE HOMOGENATES

To compare photometric determinations for sodium and potassium in the presence and absence of PO_4 in tissue homogenates rat muscle was used. The left gastrocnemius muscle was removed from 12 white male rats each weighing about 200 grams. Muscles were weighed, homogenized in 100 cc. of distilled water in the Waring blender for four minutes and divided into equal 50 cc. lots.

A. Phosphate Series. The aliquots with normal tissue phosphate contents were treated with a drop phenolphthalein, then vacuum-filtered through ash-free filter paper (S and S No. 589 Blue Ribbon) and read directly at 1/100 dilution for

TABLE 2. pH, PHOSPHATE, SODIUM AND POTASSIUM VALUES BEFORE AND AFTER CaO TREATMENT TO REMOVE PHOSPHATES

	pH		PO_4 mgm. %		POTASSIUM mgm./gram		SODIUM mgm./gram		PO_4 REMOVED
	pre CaO	post CaO	pre CaO	post CaO	pre CaO	post CaO	pre CaO	post CaO	
12 Muscles mean values	7.9	9.7	1.03	0.22	3.29	3.34	0.422	0.423	79%

TABLE 3. SODIUM AND POTASSIUM VALUES OF TISSUE ASH SOLUTIONS BEFORE AND AFTER PHOSPHATE REMOVAL

MUSCLE NO.	PRE CaO	POST CaO	PRE CaO	POST CaO
	mgm. potassium/gram		mgm. sodium/gram	
1	3.32	3.28	0.441	0.441
2	3.86	3.86	0.545	0.545
3	3.26	3.20	0.565	0.582
4	2.55	2.52	0.485	0.507
5	2.81	2.83	0.466	0.466
Mean	3.18	3.14	0.500	0.508

Our data thus indicate that in neither of the 3 cases studied—urine, tissue homogenate or tissue ash solutions—does the removal of phosphate from solutions significantly elevate or depress the sodium or potassium values obtained by flame photometry.

sodium and usually at 1/300 dilution for potassium. Aliquots of these solutions were taken for PO_4 and pH determinations.

B. Phosphate-free Series. The aliquots designed to be phosphate-free were treated with a drop of phenolphthalein, enough CaO to turn the solutions pink, vacuum filtered and analyzed concurrently with the phosphate series after pH and PO_4 determinations were made.

Table 2 indicates that sodium and potassium mean values do not show significant differences after removal of most of the phosphates.

TISSUE-ASH SOLUTIONS

To compare photometric determinations for sodium and potassium in the presence and absence of phosphates in tissue-ash solutions, gastrocnemius muscles were removed from five white male rats, weighed wet, then ashed at 200° C. for one-

half hour, then at 400 to 500° C. for two hours. The tissue ashes were taken up in 10 cc. .1 N hydrochloric acid (sodium and potassium-free) and allowed to stand for 30 minutes. Twenty cc. distilled water were added to the crucibles whose contents were then transferred to 100 cc. volumetric flasks and made up to volume with distilled water.

A. Phosphate Series. The tissue ash solutions containing phosphate were treated with 1 drop of phenolphthalein and filtered through ash-free (S and S No. 589 Blue Ribbon) filter paper.

B. Phosphate-free Series. The tissue ash solutions designed to be phosphate-free were treated with 1 drop of phenolphthalein and sufficient CaO to turn solutions permanently pink, then filtered as above and analyzed directly on the photometer concurrently with the phosphate series. Sodium values were determined in 1/100 dilution and potassium values in 1/300 dilution.

As is shown in table 3 the removal of PO_4 from tissue-ash solutions does not significantly elevate or depress the sodium or potassium values.

DISCUSSION

We believe that the discrepancy between our findings and those of Crismon may be due to the fact that our determinations were made at considerably greater dilutions than those used by him. Our sodium determinations of tissue ash were made in dilutions of 1:100 while he used more concentrated solutions. In the case of potassium determinations we used dilutions of 1:300. Crismon in a personal communication also has suggested that concentration differences may be the basis for our different findings. Berry, Chappel and Barnes (4) have reported abnormal flame photometer readings for sodium and potassium values in the presence of a variety of interfering substances including phosphoric acid and phosphate salts and marked decreases in sodium and potassium readings obtained with progressive increases of concentration of these substances. To minimize these effects and also to obtain linear calibration curves it is important to use dilute solutions.

Hald (5) in comparing sodium and potassium in aliquots of urine samples by flame photometry and by chemical procedures has found excellent agreement in the general range of dilutions similar to those we have used.

SUMMARY

Removal of phosphates by CaO precipitation does not significantly affect flame photometer determinations of sodium or potassium in urine, homogenized muscle or ashed muscle if the determinations are made on sufficiently dilute solutions and compared to dilute standards.

We express our thanks to Mr. Fred Elmadjian for making pH and phosphate determinations on many of our samples.

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HIGH LEVELS OF DIETARY POTASSIUM AND MAGNESIUM AND GROWTH OF RATS

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THE toxicity of potassium administered by stomach tube to rats as potassium chloride, potassium citrate, potassium acetate and potassium bicarbonate has been investigated by Thatcher and Radike (1). They found that the toxicity of the chloride, acetate and citrate was not essentially different while there was a lower tolerance for potassium bicarbonate. There is evidence available in the literature for the antagonistic relationship between the K^+ ion and the Ca^{++} ion in physiological processes (2, 3). Winkler, Hoff and Smith (2) found that the toxicity of injected potassium was diminished by the simultaneous injection of calcium. It is quite possible that a similar antagonistic relationship may exist between potassium and magnesium.

The data reported in this paper are the result of studies designed to give information on the relative toxicity of different potassium salts when added to the diet of rats and the effect of sub-optimum and high levels of magnesium. The latter phase of the work was undertaken to obtain information on whether or not the high potassium content of the young growing wheat plant may be a factor in the etiology of grass tetany in bovine. This malady which has been reported to occur in various parts of the world (4-6) is characterized by hypomagnesemia. In the course of studies on the etiology of the malady, it has been observed at this Station that the young wheat plant from areas where the disease is enzootic is very high in potassium containing in the order of 3.5 per cent on a dry basis. This is two to three times higher than is normally found in young herbage.

EXPERIMENTAL

Weanling rats weighing from 45 to 60 grams were used in these studies. The animals were confined to wire bottom cages and food supplied *ad libitum*. The basal diet had essentially the same composition as given in a previous publication (7). The potassium content of the basal diet on the basis of the salt mixture was calculated to be 0.58 per cent. The magnesium content was 0.015 per cent. This is a borderline level of magnesium. According to Kunkel and Pearson (7) 0.02 per cent of magnesium satisfies the requirements for optimum growth and performance of the rat. While 0.015 per cent of magnesium in the diet was adequate for growth it was not adequate to maintain normal levels of magnesium in the blood. Magnesium was

Received for publication May 3, 1948.

supplied as the sulfate and incorporated in the diet to furnish either 0.015 or 0.10 per cent of magnesium. Various potassium salts were added to the diets as indicated in table 1. The calculated amounts of calcium and phosphorus in the diet as supplied by the salt mixture were 0.75 and 0.43 per cent, respectively.

Growth. There was some mortality in some of the groups by the end of three weeks, therefore, the data on growth in table 1 for the rats on the various dietary regimens are expressed on the average gain per week over a three-week period. Some of the groups were continued for a longer period but the relative differences in the rates of gain were not essentially different.

Rats fed the basal diet containing 0.58 per cent potassium and 0.015 per cent magnesium made an average weekly gain of 30.2 grams which is significantly greater statistically than the average weekly gain of 18.6 grams when the diet contained 5

TABLE 1. TOXICITY OF POTASSIUM SALTS TO RATS

DIETARY TREATMENT			NO. OF ANIMALS ¹	MORTALITY	GAIN PER WEEK ²
Potassium		Magnesium			
% of K	salt	%		%	gm.
0.58	basal diet	0.015	24	0	30.2
5.00	KHCO ₃	0.015	26	0	18.6 ³
5.00	KHCO ₃	0.100	17	0	21.5
3.00	K ₂ CO ₃	0.015	9	0	20.0
5.00	K ₂ CO ₃	0.015	8	62	6.1
5.00	K ₂ CO ₃	0.100	18	17	10.1

¹ Number of animals at the beginning of the experiment.

² Calculated for number of animals surviving at end of the 3-week experimental period.

³ Fisher's *t* value between average weekly gain of 30.2 grams and 18.6 grams is 2.3, a significant difference. Difference in rate of gain between 18.6 grams and 21.5 grams is not significant.

per cent of potassium as potassium bicarbonate. When the diet contained 0.10 per cent of magnesium and 5 per cent of potassium the average weekly gain was 21.5 grams.

The feeding of 3 per cent of potassium as potassium carbonate had a definite depressing effect on the rate of gain. The average weekly gain on a diet containing 3 per cent of potassium as potassium carbonate was 20.0 grams as compared to a weekly gain of 6.1 grams when the level of potassium was increased to 5 per cent. At a 5 per cent level of potassium and 0.015 per cent magnesium 62 per cent of the animals died before the end of three weeks. Increasing the magnesium content of the diet containing 5 per cent potassium as the carbonate from 0.015 per cent to 0.10 per cent resulted in an increase in the average weekly gain of from 6.1 grams to 10.1 grams and a decrease in the mortality of from 62 to 17 per cent. The toxicity of potassium as the carbonate was 62 per cent at the 5 per cent level while at the 3 per cent level there were no losses during the first three weeks.

From these data it is apparent that levels of potassium of 5 per cent as the bicarbonate and either 3 or 5 per cent as the carbonate definitely reduces the rate of gain of rats. Increasing the magnesium content of the high potassium diets from

0.015 per cent to 0.10 tended to reduce the toxicity of potassium as measured by rate of gain in body weight and the mortality.

Blood Magnesium. In a few instances rats on high levels of potassium and 0.015 per cent of magnesium developed hyperemia which persisted for four or five days. The manifestations could not be differentiated from the hyperemia that characterizes a magnesium deficiency. The possibility existed that excessive levels of potassium might increase the dietary requirement for magnesium possibly through decreased absorption or increased excretion. To check this possibility the magnesium determinations were made on the whole blood of rats that had been on the various dietary regimens. The magnesium content of the whole blood was determined colorimetrically (8). In some instances it was necessary to pool blood from two or more rats so as to obtain a sufficient amount for analysis. A representative number of animals on various dietary regimens was used for the determination of blood magnesium.

TABLE 2. EFFECT OF THE LEVEL OF POTASSIUM AND MAGNESIUM IN THE DIET ON THE MAGNESIUM LEVEL OF THE BLOOD

DIETARY TREATMENT			NO. OF ANIMALS ¹	BLOOD MAGNESIUM
Potassium		Magnesium		
% of K	Salt	%		mgm. %
0.58	basal diet	0.015	7	2.4
5.00	KHCO ₃	0.015	15	2.5
5.00	KHCO ₃	0.100	16	3.0
3.00	K ₂ CO ₃	0.015	8	2.7
5.00	K ₂ CO ₃	0.100	6	3.1

¹ Due to the small amount of blood obtained from some individual animals it was sometimes necessary to pool the blood from 2 or more animals. Consequently the figures in the column headed 'number of animals' represent number of individual and/or pooled samples analyzed.

The average magnesium content of the whole blood of the rats on the basal diet containing no extra potassium and 0.015 per cent magnesium was 2.4 mg. per cent. Feeding a diet containing 5 per cent of potassium as the bicarbonate did not influence the magnesium content of the blood since the level for the animals on this treatment was 2.5 mg. per cent. There was no essential difference in the magnesium content of the blood of rats receiving potassium as the bicarbonate or as the carbonate. The mortality of the rats, fed potassium carbonate at a level to provide 5 per cent of potassium, was so high that there were insufficient animals remaining at the end of the three-week period, and the ones left were so small that it did not permit obtaining analytical data of interpretive value. It is of significance that the feeding of 0.10 per cent of magnesium in the high potassium diets did result in higher blood magnesium levels.

SUMMARY

The feeding of potassium bicarbonate at a level to furnish 5 per cent of potassium in the diet depressed the rate of growth of rats. Potassium carbonate added to the

diet at a level to furnish 3 per cent of potassium depressed the rate of gain approximately the same extent as did potassium bicarbonate at a level of 5 per cent of potassium. Five per cent of potassium as the carbonate resulted in a high mortality. Increasing the magnesium content of the high potassium diets reduced the mortality but did not significantly increase the rate of gain.

Feeding high levels of potassium had no significant effect on the amount of magnesium in the blood.

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TWO FRACTIONS OF SPECIFIC CHOLINESTERASE PRESENT IN HOMOGENIZED NORMAL MOUSE BRAIN^{1,2}

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IT HAS been reported (1-4) that the cholinesterase activity of brain is due predominantly, if not solely, to the presence of a specific enzyme, in contrast to the additional presence of a non-specific enzyme in other tissues. In the course of some measurements of the cholinesterase activity of mouse brain homogenized in a salt solution it was noted that the activity of the supernatant fluid obtained by centrifuging was considerably less than the activity of the uncentrifuged homogenate.

The factors controlling the distribution of the cholinesterase activity of mouse brain between the precipitate and the supernatant fluid have been investigated. Some chemical and physical properties of the two fractions have also been studied, in order to determine wherein the two fractions differ.

PROCEDURE

Normal white mice were killed with chloroform, and the brains were removed as quickly as possible after death. The brains were washed with 0.9 per cent NaCl solution, blotted on filter paper and weighed on a torsion spring balance, after which they were transferred to a Potter all-glass homogenizer. Five cc. of the homogenizing medium was added, and after homogenization the apparatus was washed repeatedly with the medium, the washings being added to the original homogenate. The homogenates were equivalent to a 1 to 2 per cent brain suspension.

The determinations of the cholinesterase activity of the preparations were done in the Warburg apparatus at 37°C. and in an atmosphere of 5 per cent CO₂ - 95 per cent N₂. The main compartment of the vessel contained 2 cc. of the enzyme preparations plus 0.8 cc. of an appropriate salt solution which varied according to the composition of the homogenate; the side arm contained 0.2 cc. of the substrate. The final concentrations of salts after tipping the vessels usually were: NaCl 0.15 M, MgCl₂ 0.04 M, and NaHCO₃ 0.025 M. The substrate was usually acetylcholine chloride in a final concentration of 0.015 M.

The precipitate, obtained by centrifugation (for 20 minutes with a 'g' value between 900 and 2000) and decantation, was transferred to the homogenizer and resuspended by homogenizing again in the appropriate medium. The volume of the resuspended precipitate homogenate was equal to that of the original uncentrifuged homogenate.

The results are expressed as mgm. substrate hydrolyzed per 100 mgm. fresh tissue per hour (QA·Ch.).

RESULTS

Distribution of cholinesterase activity in a 'dilute' buffer homogenate. The results are given in table 1. The brain was homogenized in the buffer and the supernatant

Received for publication May 12, 1948.

¹ Presented in part before the American Physiological Society, Atlantic City, 1948. *Federation Proc.* 7: 73, 1948.

² Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

fluid and precipitate were separated by centrifuging. It will be seen that approximately 83 per cent of the total activity was present in the precipitate.

Distribution of cholinesterase in a distilled water homogenate. The results are given in table 1. The brain was homogenized in distilled water, and the homogenate

TABLE 1. PARTITION OF CHOLINESTERASE ACTIVITY IN 'DILUTE' BUFFER AND DISTILLED WATER HOMOGENATES¹

EXP. NO.	BUFFER HOMOGENATE			WATER HOMOGENATE	
	Total homogenate Q.A.Ch.	Supernatant activity	Precipitate activity	Total homogenate Q.A.Ch.	Supernatant activity
		%	%		%
1	8.71	15.8	85.9	8.10	86.7
2	8.64	17.3	81.8	8.75	87.3
3	9.00	23.3	70.6	8.85	88.9
4	9.43	15.2	79.2	9.13	92.7
5	8.38	16.5	87.5	9.57	89.2
6	8.48	16.2	84.3	8.60	92.4
7	9.01	15.0	85.2	8.67	89.7
8	8.39	19.0	87.5	6.68	87.4
9	9.06	15.4	82.5	11.25	95.1
10	9.11	14.5	84.7		
Mean	8.82	16.8	82.9	8.84	89.8

¹ 'Dilute' buffer concentration: NaCl 0.15 M, MgCl₂ 0.04 M, NaHCO₃ 0.025 M; substrate: acetylcholine chloride 0.015 M in 'dilute' buffer.

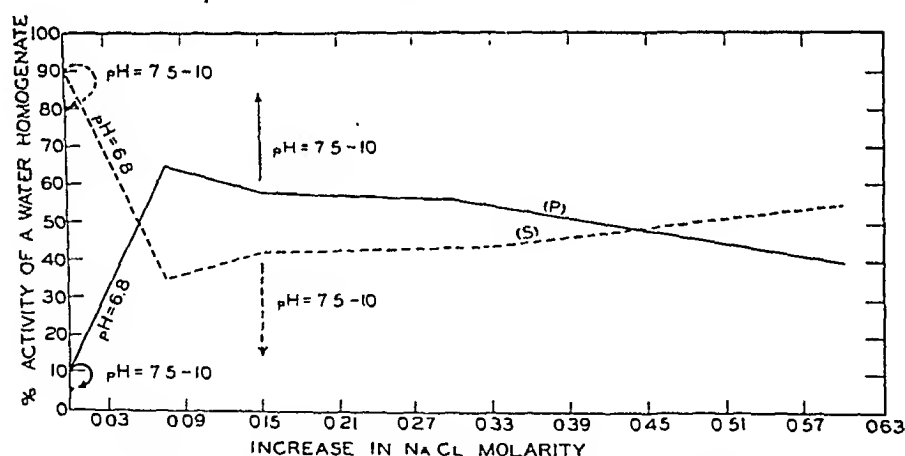


Fig. 1. EFFECT OF CHANGES in NaCl molarity and pH on the distribution of cholinesterase. S, supernatant fluid; P, re-homogenized precipitate.

was centrifuged. The supernatant was decanted. The final desired concentration of salts was obtained by adding to the Warburg vessels 0.8 cc. of 'medium' buffer (NaCl 0.525 M, MgCl₂ 0.14 M, NaHCO₃ 0.0875 M). It will be seen that approximately 90 per cent of the total activity was present in the supernatant fluid.

Effect of changing the pH and the NaCl molarity on the cholinesterase distribution. The results are given in figure 1. A water homogenate was prepared, and the NaCl

concentration of aliquots of the original homogenate was varied by the addition of appropriate NaCl solutions. An aliquot of the original homogenate was also diluted by the addition of an equivalent volume of distilled water. The homogenates were centrifuged, and the cholinesterase activities of the supernatant fluids and re-homogenized precipitates were determined. It will be seen that 90 per cent of the total water homogenate activity was present in the supernatant fluid, but that as the NaCl concentration was increased the enzyme activity of the supernatant fluid decreased and remained relatively constant over a wide range of NaCl concentration. The maximum decrease in the enzyme activity of the supernatant fluid was reached with an NaCl concentration of 0.075 M. As the enzyme activity of the supernatant

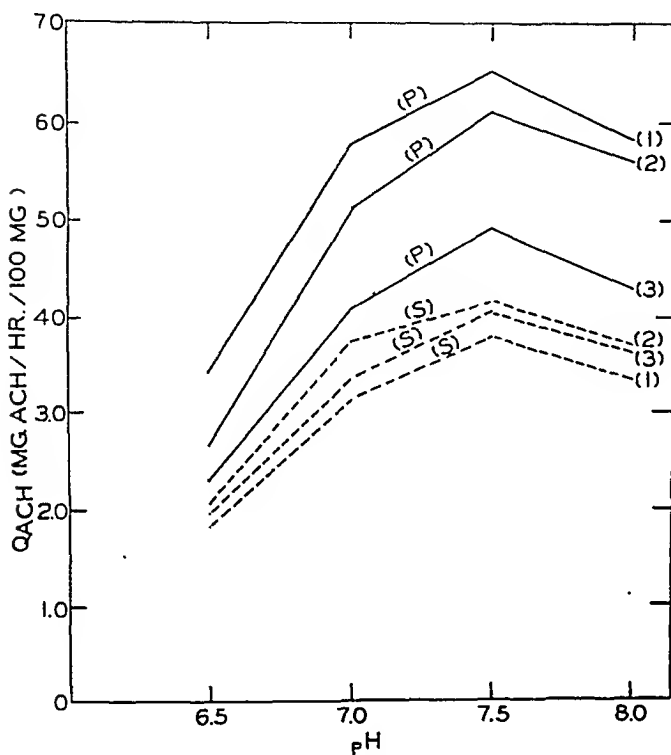


Fig. 2. ACTIVITY OF THE CHOLINESTERASE FRACTIONS determined at various pH values (3 experiments). A distilled water homogenate was adjusted to 0.15 M with NaCl before centrifuging. - - -, supernatant fluid; — re-homogenized precipitate.

fluid decreased, the cholinesterase activity of the precipitate increased an equivalent amount.

When the pH of a water homogenate was varied between 7.5 and 10 by the addition of 0.1 N NaOH (0.05–0.2 cc. per 12 cc. of homogenate) the enzyme activity of both the supernatant fluid and the precipitate decreased slightly, but there was no change in the distribution of cholinesterase.

When the pH of a 0.15 M NaCl homogenate was varied between 7.5 and 10, in the same manner as above, the cholinesterase activity of the precipitate was increased from 59 per cent of the total activity to 84 per cent, while the activity of the supernatant fluid was decreased from an original value of 41 per cent to a value of 15 per cent.

Effect of pH upon the activity of the cholinesterase fractions. The results are given in figure 2. Distilled water homogenates were prepared and NaCl was added

until the increment of concentration was 0.15 M. The two fractions were separated by centrifuging, and the activities were determined. The pH of the reaction mixture was varied by adjusting the NaHCO_3 concentrations, keeping the $p\text{CO}_2$ constant. It will be seen that the patterns of enzyme activity were quite similar for the supernatant fluid and the precipitate. The optimum pH was approximately 7.5.

*Activity patterns of the cholinesterase fractions towards various substrates*³. The results are given in figure 3. It will be seen that there was no significant difference between the activity patterns of a 'dilute' buffer homogenate, the supernatant fluid, and the precipitate derived from it (A). This is the activity pattern reported by Nachmansohn and Rothenberg (3) for specific cholinesterase.

When the supernatant fluid from a water homogenate, which was adjusted to 0.075 M with NaCl before centrifuging, was concentrated several times by vacuum

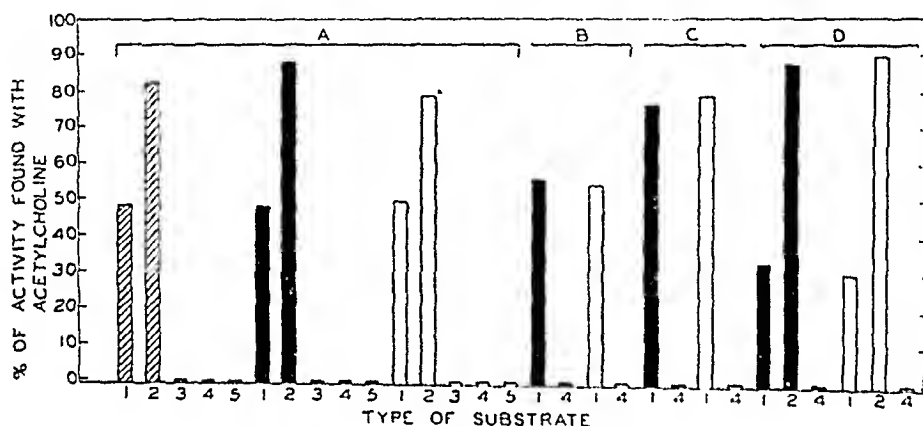


Fig. 3. ACTIVITY PATTERN OF THE CHOLINESTERASE FRACTIONS with various substrates. ▨ uncentrifuged homogenate, ■ supernatant fluid, □ re-homogenized precipitate. A, 'dilute' buffer homogenate; substrate concentration 0.015 M. B, C, D, distilled water homogenate adjusted to 0.075 M with NaCl; the supernatant fluid was concentrated 3.2 to 4.4 times by vacuum distillation at 39-42°C; substrate concentrations: B—0.015 M, C—0.045 M, D—0.0015 M. Substrates: 1—acetyl-β-methylcholine, 2—propionylcholine, 3—butyrylcholine, 4—benzoylcholine, 5—ethyl butyrate.

distillation, it was found not to differ significantly from the corresponding precipitate at three levels of substrate concentration (B, C, D). It will be seen, however, that the activity patterns of both enzyme fractions varied considerably depending upon the substrate concentration.

Effect of heating upon the activities of the cholinesterase fractions. The results are given in figure 4. The supernatant fluid and precipitate fractions were obtained by centrifuging a distilled water homogenate adjusted to 0.15 M with NaCl or by centrifuging 'dilute' buffer homogenates. Aliquots of these fractions were heated in a constant temperature water bath at 50°, 54°, and 60°C. for 15, 30, 45, and 60 minutes. The enzymic activities of the heated aliquots were compared with the unheated aliquots. Heating at 60°C. resulted in complete disappearance of cholinesterase activity in both fractions within 15 minutes. It will be seen that at 50°C.

³ The benzoylcholine chloride, propionylcholine chloride and butyrylcholine chloride were supplied by Dr. E. L. Sevringhaus of Hoffman-La Roche, Inc., Nutley, N. J.

there was some decrease in the enzymic activities of both fractions, but it is questionable if there is a significant difference between the two. At 54°C. there was a decreased activity in both fractions, but the activity of the precipitate decreased considerably more than the supernatant fluid activity.

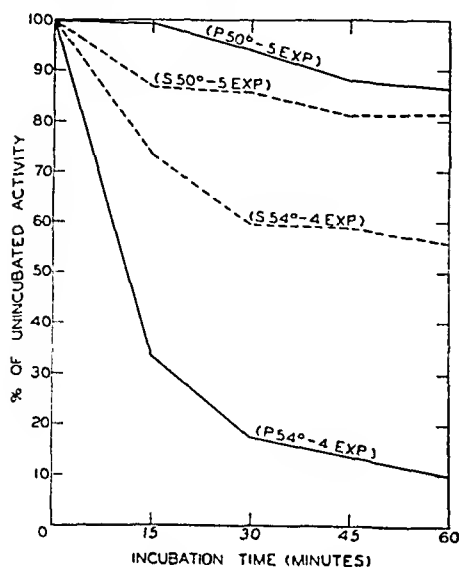


Fig. 4. EFFECT OF HEATING on the activities of the cholinesterase fractions from dilute buffer and distilled water homogenate adjusted to 0.15 M with NaCl. - - - supernatant fluid, — re-homogenized precipitate.

DISCUSSION

It was thought that the difference in the distribution of cholinesterase between the 'dilute' buffer homogenate and the distilled water homogenate might be explained by the presence of intra- and extra-cellular fractions of the enzyme with incomplete cell disruption, and therefore precipitation on centrifuging the isotonic buffer homogenate. However, it was found that when a 'dilute' buffer homogenate was subjected to alternate freezing with CO₂ ice and thawing or when the homogenate was prepared by grinding with alundum, two procedures which should complete cell disruption, there was no change in the enzyme distribution. It was concluded that the distribution difference did not depend upon the presence of intact cells.

The two most obvious differences in the composition of the two types of homogenates were the NaCl concentration and the pH. The pH of the 'dilute' buffer homogenate had an average value of 8.5, while the average pH value of the distilled water homogenates was 6.8. Upon varying the NaCl concentration of a water homogenate, it was found that the distribution of the enzyme fractions was changed, but the distribution was not quite equivalent to that found in the buffer homogenate. When the pH of a water homogenate was varied alone, there was no change in the distribution of the enzyme, but apparently there was some slight enzyme inactivation in both the supernatant fluid and precipitate fractions. However, when the pH of a 0.15 M NaCl homogenate was varied, it was found that the distribution of the enzyme was equivalent to that found in a buffer homogenate. It is concluded that basically the distribution is dependent upon the NaCl concentration, but that varying the pH of the medium has an additional effect.

Mendel and Rudney (1) reported that when mouse brain was ground and suspended in about 5 volumes of distilled water and centrifuged, the precipitate contained the cholinesterase. These results appear to be at variance with the results reported here. However, their preparation was a 20 per cent suspension while our preparation was a 1 to 2 per cent suspension, and it can be calculated that the NaCl concentration in their distilled water preparation was approximately 0.03 M while ours was approximately 0.003 M, assuming a tissue concentration of 0.15 M NaCl.

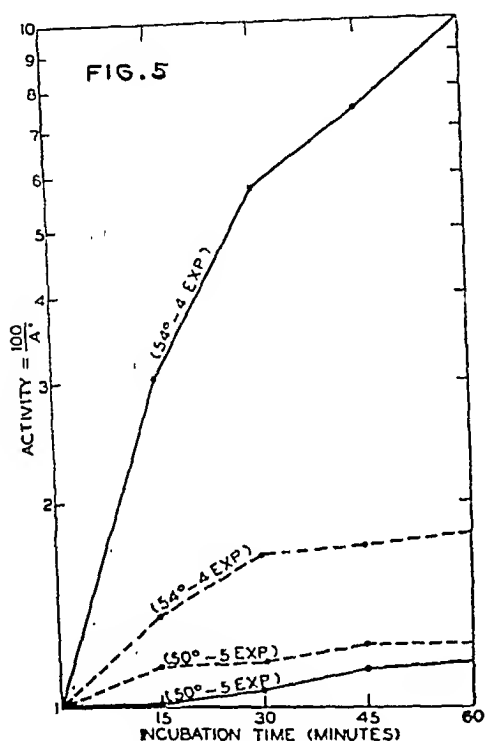


Fig. 5. RELATIONSHIP between $\log \frac{A}{A'}$ and t . A , initial unheated activity; A' , activity at t time. - - - supernatant fluid; — re-homogenized precipitate.

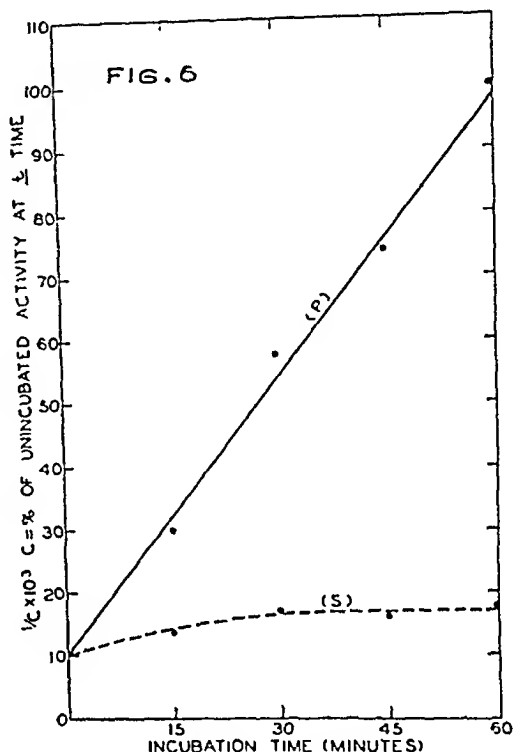


Fig. 6. RELATIONSHIP between $\frac{1}{c}$ and t . c , percentage of unheated activity at t time. - - - supernatant fluid; — re-homogenized precipitate.

It will be seen from figure 1 that the maximum precipitation occurred at a NaCl concentration between 0.03 and 0.075 M, and that at 0.03 M NaCl 35 per cent of the activity was found in the precipitate. It appears that the effect of NaCl concentration on the enzyme distribution is quite critical in this range of concentration, and it may be that this fact will explain the apparent discrepancy between our observations and those of Mendel and Rudney.

There was no difference in the activity pattern of the two fractions of cholinesterase against the various substrates. There was some doubt that the true activity against butyrylcholine, benzoylcholine, and ethyl butyrate could be determined in the supernatant fluid from a 'dilute' buffer homogenate (fig. 3A), because the average $Q_{A \cdot Ch}$ was 1.6. For this reason, the supernatant fluid was concentrated several

times (fig. 3B), and the activity against the above three substrates was found to be zero. It is of interest that the activities of the two enzymes towards acetyl- β -methylcholine relative to the activity towards acetylcholine are quite dependent upon the substrate concentration (fig. 3B, C, D), although this does not appear to be the case with propionylcholine (fig. 3A, D).

In addition to the effects of the NaCl concentration and pH upon the enzyme distribution, the only other difference found between the two cholinesterase fractions was their heat labilities.

In order to determine whether the heat inactivation of the two fractions of the enzyme followed any known reaction order, the data were plotted according to the following two methods. a) Using the formula $k = \frac{1}{t} \log \frac{A}{A'}$,

where A is the initial unheated activity (100 per cent) and A' is the relative activity at t time, if one plots the $\log \frac{A}{A'}$ against t , as in figure 5, it will be seen that none of the relationships give a straight line function, which is contrary to what would be expected if the inactivation were a first order reaction. b) However, plotting the reciprocal of the activity at t time against t does give a straight line function for the precipitate at 54°C. (fig. 6). This is the relationship that would be expected of a second order reaction. Assuming that these expressions of activity are adequate, it would appear that the heat inactivation of the enzyme in the precipitate may be a second order reaction.

The fractional distribution of the cholinesterase activity in homogenized mouse brain reported here may be interpreted as indicating the presence of two distinct specific enzymes, the presence of two degrees of aggregation of the same enzyme, or a difference in the adsorption of the enzyme on some other molecule. Although the heat lability studies may favor the presence of two distinct specific enzymes in the homogenate, it is believed that the available data are inconclusive in differentiating between the above three possibilities.

SUMMARY

It has been shown that in homogenized mouse brain the specific cholinesterase activity can be separated by centrifugation of the homogenate into two fractions. One of these fractions is precipitated maximally from a distilled water homogenate when the concentration of NaCl is adjusted to 0.075 M. Increasing the pH of a NaCl homogenate up to pH 7.5–8.0 causes a further precipitation of this fraction.

The distribution of the activity in the precipitate and supernatant fluid fractions is not dependent upon the presence of intact cells, and therefore cannot be due to the presence of intra- and extra-cellular fractions. The activities of the two fractions have the same pH optima; and there is no difference in the relative activities of the two fractions towards acetyl- β -methylcholine, propionylcholine, butyrylcholine, benzoylcholine and ethyl butyrate. Upon heating at 54°C., the precipitated fraction is considerably more heat labile than the supernatant fraction. It appears that the heat inactivation of the precipitated fraction is a second order reaction.

The author acknowledges with appreciation the assistance of Miss Earline Tapp in many of these experiments.

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NONEXCRETION OF JAUNDICE-SERUM ALKALINE PHOSPHATASE IN BILE OF NORMAL DOGS

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THERE is no unanimity of opinion regarding the origin of the increased alkaline phosphatase of the blood serum in obstructive jaundice. A large number of studies of various types have yielded data that have been interpreted as indicating that it originates *a*) entirely in the skeleton, or *b*) partly in the liver. The prevailing view is that, whatever its origin may be, the increase in serum alkaline phosphatase activity that follows biliary obstruction is due to blocking of its normal excretory channel, i.e., the bile duct system.

Freeman and Chen (1) transfused normal dogs with blood of high phosphatase activity, obtained from dogs 10 to 15 days after ligation of the common duct. The increased phosphatase activity in the blood of the recipients persisted for several days, falling to normal levels only after 5 to 7 days. The authors interpreted this slow disappearance of injected phosphatase as indicating either that most of the normal phosphatase of bile does not come from the plasma or that the phosphatase in jaundiced blood is bound to some substance which retards its excretion by the hepatic cells. The present study was undertaken for the purpose of determining whether an excess of circulating phosphatase of this nature (obstructive jaundice) can be excreted in the bile of dogs with normally functioning livers.

METHODS

Heparinized plasma obtained from 3 dogs 10 days after cholecystectomy and ligation of the common bile duct was injected intravenously (in 15 minutes) in three trained Thomas-type duodenal fistula dogs (2, 3) in quantities of about 10 per cent of the estimated blood volume, the exact amounts being indicated in table 1. No untoward reactions were observed. Bromsulfalein excretion tests (4) yielded normal results 24 hours before and 72 hours after the plasma transfusion in dogs 2 and 3; this test was not performed in dog 1.

Bile was collected by direct cannulation of the orifice of the common duct through the duodenal fistula (4) in 1-hour samples, from 1 hour before to 4 hours after beginning the plasma infusion. In dogs 2 and 3, additional one-hour bile samples were collected during the periods 24 to 25 hours and 48 to 49 hours after the plasma infusion. Specimens of blood were obtained before, and 1, 2, 3, 4, 24 and 48 hours after commencing the infusion. Alkaline phosphatase activity was determined in the serum and bile specimens and in the infused plasma by the method of Bodansky (5).

Received for publication June 3, 1948.

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RESULTS AND DISCUSSION

The pertinent data are presented in table 1.

The persistent elevation of serum phosphatase activity in the recipient dogs is in agreement with the findings of Freeman and Chen (1). It is of interest that the degree of elevation in each instance was greater than can be accounted for by the phosphatase activity of the injected plasma. This observation is in accord with reports by Thannhauser *et al.* (6) and Cantarow (7) that normal serum phosphatase is 'activated' by addition *in vitro* of serum of high phosphatase activity (obstructive jaundice and Paget's disease).

During the first hour after infusion of the plasma, the concentration of phosphatase in the bile rose from the control levels of 17 to 22 units to 110 to 191 units per 100 ml., falling during the second hour to 38 to 46 units, the latter levels being maintained

TABLE 1. ALKALINE PHOSPHATASE ACTIVITY (BODANSKY UNITS) IN BLOOD AND BILE BEFORE AND AFTER INTRAVENOUS INFUSION OF PLASMA OF HIGH PHOSPHATASE ACTIVITY

DOG	AMOUNT INJECTED		BLOOD SERUM								BILE							
			Hours after starting injection								Hours after starting injection							
			0	1	2	3	4	24	48		-1 to 0	1	2	3	4	24-25	48-49	
1 15 kgm.	144 ml. (225 U)	Units/100 ml.	1.6			42.9	41.4	23.8	18.9	ml. Total units	1.2 0.2	1.1 2.1	1.6 0.6	0.5 0.2	1.7 0.6			
2 12 kgm.	110 ml. (156 U)	Units/100 ml.	3.4	42.8	40.6	40.2	40.4	28.3	20.6	ml. Total units	2.3 0.4	2.1 2.3	1.3 0.6	1.4 0.5	0.8 0.4	1.2 0.6	1.6 0.5	
3 16 kgm.	152 ml. (153 U)	Units/100 ml.	2.1	34.3	33.6	32.4	32.0	20.3	16.4	ml. Total units	1.8 0.4	2.4 3.4	1.8 0.7	1.6 0.6	1.2 0.5	1.6 0.5	1.7 0.5	

in subsequent observations (29 to 50 units per 100 ml.). Despite this increase in concentration, the increase in quantity of phosphatase excreted in the bile during the experimental period was comparatively insignificant (table 1). These observations indicate that alkaline phosphatase of the type that accumulates in the blood in obstructive jaundice is not removed from the blood for biliary excretion by a normally functioning liver in significant amounts. It is interesting that the rate of disappearance of this enzyme from the blood is similar to that of isotopically labelled plasma protein fractions injected into normal dogs.

These data appear to render untenable the view, originally proposed by Armstrong and Banting (8), that the increased serum phosphatase in obstructive jaundice originates in the bones and that its accumulation in the blood in this condition is due to interference with its normal excretion in the bile. If this enzyme were of extrahepatic origin, one would anticipate its excretion by a normal liver at a rate paralleling the rapidity with which it increases in the blood following biliary obstruction. By exclusion, these findings support the hypothesis that the increased serum alkaline phosphatase of obstructive jaundice arises by regurgitation from liver cells and/or

bile duct epithelium. Although histologic evidence suggests that it is capable of producing large quantities of alkaline phosphatase, the normal liver appears to be incapable of removing it from the blood in significant amounts.

SUMMARY

Blood plasma with high alkaline phosphatase activity, obtained from dogs with common bile duct obstruction, was injected intravenously in three dogs with Thomas-type duodenal fistulas. The serum phosphatase remained at a high level in the recipient dogs for at least 48 hours and very little of the infused phosphatase was excreted in the bile. These data indicate that a normally functioning liver does not actively remove phosphatase of this type from the blood and, therefore, contradict the hypothesis that it originates in extrahepatic tissues.

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INHIBITION BY HISTAMINASE OF GASTRIC SECRETION IN DOGS

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SINCE shortly after the discovery of histaminase by Best (1) in 1929, attempts have been made to counteract the action of administered or endogenously-formed histamine in man and animals by the concurrent administration of histaminase preparations. From the many studies in this field no unequivocal evidence indicating that the administration of histaminase preparations counteracts the pharmacological actions of histamine has emerged.

Several studies of the effect of histaminase preparations on histamine-stimulated gastric secretion in dogs have been made. All (2-4) except the most recent investigators, Rosterfer and Laskowski (5), failed to note any depression of the secretory response by the histaminase preparations which they used, even when these were given intravenously. Even in the case of the latter workers the depression of secretion could not be attributed to the histaminase in their preparations.

Laskowski (5) prepared a histaminase extract from hog kidneys which was considerably more potent than those used by previous workers. This material was used in the experiments of Rosterfer and Laskowski (5), who observed marked depression of the gastric acid secretion in response to histamine in Heidenhain pouch dogs. However, the material which they used produced severe toxic reactions in the dogs consisting of trembling of the extremities, coldness, ischemia of the gastric mucosa, vomiting and defecation. Since injection of a dose of this material in which almost all of the histaminase activity had been destroyed by exposure to acid reproduced these same toxic symptoms as well as the depression of the gastric response to histamine, the authors were unable to conclude that the inhibition of secretion was due to the histaminase activity of the preparation.

Recently a potent histaminase preparation¹ of low toxicity became available to us. Our studies of its effect on gastric secretion form the subject of this report.

Histaminase Powder. The histaminase preparation used in these studies is made from hog kidney. It is a light tan lyophilized powder containing approximately 40 per cent protein. The histaminase potency as measured by the Warburg manometric technique (6) is about 0.05 U/mgm. of dry powder for the average batch. Laskowski *et al.* (6) have defined this unit as the amount of enzyme which under the experimental conditions (total volume of liquid 3 ml., 1 mgm. histamine dihydrochloride, pH 7.2, 38° C., air) will utilize 1 μ l. of oxygen per minute, roughly corresponding to the destruction of 1 mgm. of histamine dihydrochloride per hour. The old Winthrop unit of histaminase is therefore about 1/24th as large as the present unit. Since the old Winthrop Torantil preparations contained about one Winthrop unit per 15 milligrams of powder or 0.0028 Warburg

Received for publication June 7, 1948.

¹ Prepared by Dr. Alex Lesuk of the Sterling-Winthrop Research Institute.

u/mgm., the preparation used in the present study is about 20 times as potent. Inasmuch as less than half of the dry weight of this new material is protein (the remainder consisting mainly of phosphate buffer and sucrose), this ratio of relative potency on the basis of protein content would be more nearly 40 to 1.

Toxicity data supplied to us by the Sterling-Winthrop Research Institute indicate that no acute toxic manifestations occurred when as much as 5000 u/kgm. of body weight was injected intravenously in mice.

PROCEDURE AND RESULTS

Effect on Histamine-Stimulated Gastric Secretion. Eight dogs with vagotomized pouches of the entire stomach were used. Histamine dihydrochloride in a dose of 0.0125 mgm. was injected subcutaneously every 10 minutes during the entire course of the experiment (5 to 7 hours). Gastric juice samples were collected, measured and titrated every 20 minutes. After one to two hours the gastric secretory response attains a relatively fixed level and maintains this level for many hours unless some inhibitory influence is interposed. This is illustrated by the control experiments in

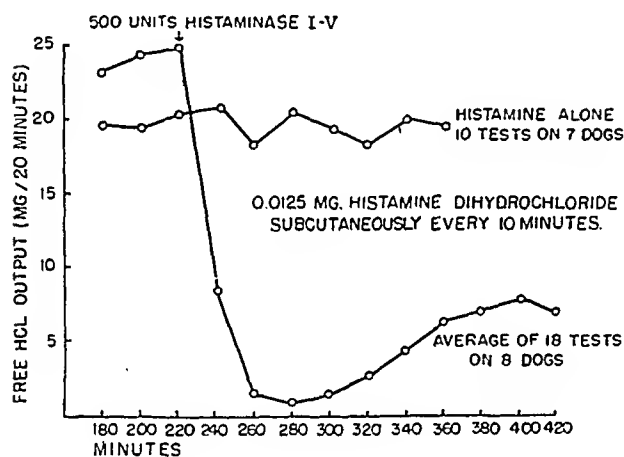


Fig. 1. AVERAGE SECRETORY RESPONSE of dogs with pouches of entire stomach to histamine alone and to histaminase plus histamine.

which only histamine was given during the entire course of the experiment in 10 tests on 7 dogs (fig. 1). In 18 other tests on 8 dogs 500 units² of histaminase was injected intravenously when the secretory rate had become stabilized (220 minutes after beginning the injections of histamine every 10 minutes). A marked depression of acid secretion ensued, the maximal effect usually occurring about 40 minutes after the injection. At the height of the effect the average inhibition was 95 per cent of the prehistaminase control level. In many instances free acid was absent from the gastric juice for a period of several hours. Three hours after the injection, on the average, the inhibition still was greater than 60 per cent. In the tests in which the observations were continued until the secretory response returned to the control level, this was usually found to require from 3 to 8 hours.

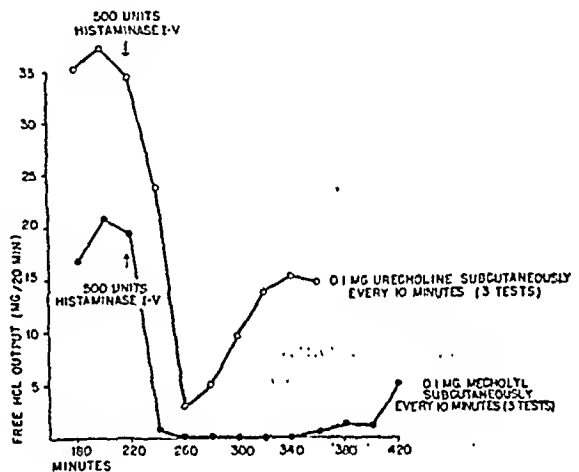
In 4 tests on 4 dogs 15 u/kgm. body weight of histaminase failed to produce inhibition, whereas in 4 other tests in which 25 u/kgm. was injected inhibition occurred in every dog, the average maximal inhibition amounting to 90 per cent. This indi-

² All doses are given in Winthrop units.

cates that the minimal effective dose for the inhibition of a histamine stimulus of the size used in these experiments (0.0125 mgm. histamine dihydrochloride every 10 minutes) is about 20 U/kgm.

Effect on Other Stimuli For Gastric Secretion. The effect of histaminase upon the response to stimuli other than administered histamine has also been studied. The same dogs with pouches of the entire stomach were used and the plan of the experiment was the same except that the parasympathomimetic drugs acetyl beta-methylcholine (Mecholyl) and carbamoyl beta-methylcholine (Urecholine) in a dose of 0.1 mgm. subcutaneously every 10 minutes were substituted for the histamine stimulus. Three tests on 3 dogs were performed using Mecholyl and three tests on 3 dogs with Urecholine. Inhibition was produced by 500 units of histaminase intravenously in every test. The average results appear graphically in figure 2.

Fig. 2. EFFECT OF HISTAMINASE ON the gastric secretory response to parasympathomimetic drugs Urecholine and Mecholyl in dogs with pouches of entire stomach.



The effect of histaminase on the gastric secretory response to a meal was tested in a Pavlov pouch dog. In two control experiments 92.1 and 47.9 mgm. of free HCl were secreted in the 3-hour period following a 150-gram meal of ground horse meat. In three similar experiments 500 units of histaminase was given intravenously immediately after feeding the meat; the output of free HCl in these experiments was 8.1, 14.9 and 5.3 mgm. for the 3-hour test period.

Heat Stability. When a solution containing 100 mgm. of histaminase powder per cc. of 0.9 per cent sodium chloride solution is heated in a water bath to 60° C. for five minutes, a heavy flocculent precipitate forms. The supernatant fluid separated by centrifugation still possesses gastric secretory depressant activity. The results of three tests on 3 dogs in which this heat-fractionated supernatant was used appear in figure 3. It will be noted that the inhibition of gastric secretion produced by this heat-fractionated supernatant is essentially the same as that produced by the original material. The supernatant of the heat-fractionation process can be dried by lyophilization and then redissolved before use. Due to technical difficulties in lyophilization some of our batches of heat-fractionated histaminase were inactivated during drying. It is interesting to note that those batches which were devoid of histaminase activity were also ineffective in depressing gastric secretion. This correla-

tion between loss of histaminase activity and loss of gastric secretory depressant activity is revealed by the data in table 1.

Heating of the histaminase solutions in a boiling water bath for one minute results in complete destruction of both the histaminase activity and the gastric secretory depressant activity (fig. 3).

Toxicity. General toxicity is minimal. The material as supplied to us produces slight retching and vomiting when given intravenously in doses of 50 U/kgm. This occurs within one or two minutes after completing the injection and then subsides in

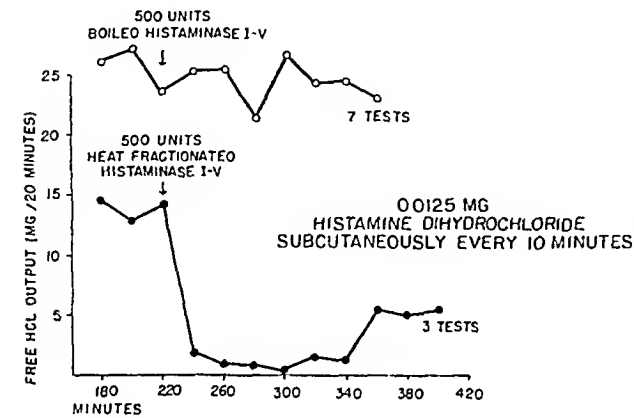


Fig. 3. EFFECT OF EXPOSURE of histaminase solution to a temperature of 60° C. (heat-fractionated) and 100° C. (boiled) for 5 minutes on its inhibitory action on gastric secretion.

TABLE 1. HISTAMINASE ACTIVITY AND GASTRIC SECRETORY DEPRESSANT ACTIVITY OF VARIOUS LOTS OF HEAT-PURIFIED HISTAMINASE

LOT NO.	POTENCY OF STARTING MATERIAL	POTENCY OF HEAT-PURIFIED LYOPHILIZED HISTAMINASE	GASTRIC SECRETORY DEPRESSANT ACTION (3 TESTS ON 3 DOGS)
	Warburg U per mgm.		
1	0.028	0	0
2	0.028	0	0
3	0.028	0.019	+
4	0.028	0.028	+
5	0.05	0.030	+
6	0.05	0.030	+
7	0.05	0	0

another one or two minutes. Injection of the supernatant of the thermal fractionation at 60°C. (see above) produces no retching, vomiting or any other obvious side effect. This indicates that the retching and vomiting are not due to the histaminase and that the depression of gastric secretion is not due to the retching and vomiting.

Intramuscular injection of the histaminase solution results in necrosis of the tissues in dogs and rabbits.

Effect on Blood Pressure. The original material contains a potent vasopressor substance. Figure 4 is a record of the vasopressor response of a cat to 50 units of histaminase given intravenously. The supernatant of the thermal fractionation procedure usually produces a slight depressor response (fig. 4). Inasmuch as this his-

taminase product is prepared from renal cortex, it is probable that the vasopressor substance is renin.

In the anesthetized dog or cat, treatment with 100 U/kgm. of histaminase does not decrease the depressor response to minimally effective doses of histamine (10 experiments on dogs, 3 on cats). Larger doses of histaminase (1000 U/kgm.) definitely inhibit the vasodepressor action of histamine in cats (7).

Antigenicity. Both the original material and the heat-purified product are strongly antigenic. Table 2 is the protocol of an experiment on the production of anaphylaxis in guinea pigs using the heat fractionated material.

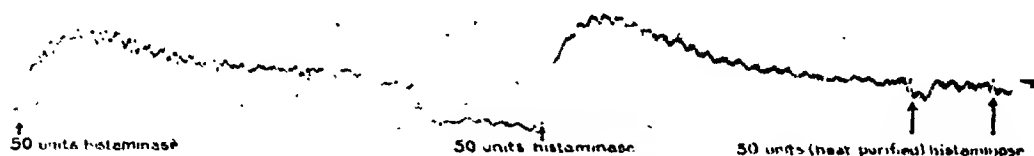


Fig. 4. EFFECT OF HISTAMINASE on blood pressure of an anesthetized cat. Heat purified histaminase is the supernatant of a solution exposed to 60° C. for 5 minutes.

TABLE 2. ANTIGENICITY OF HEAT-FRACTIONATED HISTAMINASE

GUINEA PIG	WT. gm.	SENSITIZING DOSE		SHOCKING DOSE		RESULT
		U	cc.	U	cc.	
1	456	100	I	100	I	Anaphylactic death
2	564	100	I	100	I	" "
3	492	100	I	100	I	" "
4	512	100	I	100	I	" "
5	548	100	I	100	I	" "
6	502	None (control)		100	I	No reaction
7	468	None (control)		100	I	" "

The sensitizing dose was administered subcutaneously and repeated 3 times at 2-day intervals. The shocking dose was given intravenously into the exposed jugular vein 3 weeks after the last sensitizing dose.

DISCUSSION

The statement has frequently been made that histaminase could not be expected to be active *in vivo* because it acts so slowly. The speed with which an enzyme acts, i.e., the amount of substrate it destroys per unit time, depends, among other things, upon the concentration of the enzyme. Therefore the 'slowness' of action of the earlier preparations was essentially a reflection of their poor state of purification or low activity per unit weight of material.

We found that about 20 U/kgm. histaminase was required to counteract the stimulating action of 0.0125 mgm. of histamine dihydrochloride given every 10 minutes. In a dog weighing 10 kgm. the total dose of histaminase would be 200 units, an amount of enzyme capable of destroying *in vitro* 200 mgm. of histamine dihydrochloride in 24 hours or 8.3 mgm. per hour. Since the histamine dihydrochloride was actually being administered at a rate of 0.075 mgm. per hour, the efficiency of the histaminase

in vivo was only $\frac{0.075}{8.3}$, or less than one hundredth of its *in vitro* potency. This calculation is based upon the assumption that all of the histamine administered is being destroyed, and if all of it is not being destroyed the efficiency ratio would be even lower.

A number of investigators (13) have found that a marked rise in the histaminolytic power of the blood occurs during pregnancy in human beings. During the second trimester one ml. of serum of a pregnant woman will, on the average, destroy 5 micrograms of histamine base per hour, which is equivalent to 0.2 Winthrop U/ml. We have not yet determined whether the histaminolytic power of the blood serum is raised by injection of the histaminase preparation we have used. However, if we assume that all of the injected histaminase remains active and that it is distributed only in the blood plasma, then a dose of 20 Winthrop U/kgm. (the minimal effective dose for inhibition of gastric secretion) would produce a serum level of about 0.4 U/ml. Little is known about the physiological significance of the rise in blood histaminase during pregnancy, but it is of interest to speculate on its possible relationship to the depression of gastric acid secretion (14) and the remission of peptic ulcer symptoms (15) which are known to occur during pregnancy.

The parietal cell is probably more sensitive to stimulation by histamine than any other body cell. We (8) have recently determined the threshold dose for stimulation of gastric secretion in the unanesthetized dog and found an average value of 0.05 micrograms of histamine base per kgm. per minute. The threshold for vaso-depressor effects in anesthetized cats and dogs has been found to be about 0.3 micrograms of histamine base per kgm. per minute (9), or six times as great as the threshold for gastric secretion.

The most important question which arises in regard to this work is whether the gastric secretory depressant activity is due to the histaminase or to some other constituent of the extract. The most important piece of evidence now available on this question is the parallelism between the heat stability of the histaminase activity and that of the gastric secretory depressant activity. This indicates that the histaminase factor and the secretory depressant factor have the same degree of thermal stability, but it does not prove that they are identical. Such an identification will be furthered by purification of the extracts and demonstration that the histaminase and secretory depressant activities are enriched to an equal degree. The final proof of their identity or lack of identity will await the isolation of the substance (or substances) in chemically pure form. Studies on the specificity of histaminase for counteracting the effects of histamine *in vivo* in organs other than the stomach should also give important evidence on this question. *In vitro* studies on the ability of this kidney extract to destroy pharmacological stimuli other than histamine, for example, acetylcholine, would be of interest.

If subsequent work does prove that the gastric secretory depressant in these extracts is indeed histaminase, then the fact that these extracts counteract the gastric secretory response not only to histamine but to food and to other drugs as well will constitute strong evidence in favor of the view that histamine may be a final common local excitant for gastric acid secretion produced by any type of stimulus. This rôle

for histamine has been suggested by several workers (10-12) in this field. This hypothesis is based mainly on the fact that histamine is usually present in the gastric juice itself, regardless of what stimulus is used to provoke the secretion. The results of the present study are suggestive but cannot yet be construed as evidence in favor of this hypothesis, because, as was pointed out above, we cannot be sure that the gastric secretory inhibition was due to the histaminase.

SUMMARY

A preparation of histaminase made from hog renal cortex and containing about 1.2 Winthrop units of histaminase per mgm. of dry powder has been tested for its gastric secretory depressant action. Twenty u/kgm. body weight given intravenously markedly inhibits the gastric secretory response to 0.0125 mgm. of histamine dihydrochloride given subcutaneously every 10 minutes in dogs with pouches of the entire stomach. No toxic side reactions occur. The secretory response to food and to parasympathomimetic drugs is also inhibited by this histaminase preparation.

A decision cannot yet be made as to whether the inhibition of gastric secretion is due to the histaminase or to some other constituent of these extracts.

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UROPEPSIN OUTPUT IN CATS TREATED WITH CAFFEINE AND HISTAMINE

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IT IS known that a functioning gastric mucosa is necessary for the presence in urine of a pepsin-like enzyme, uropepsin. After surgical destruction of the gastric mucosa in the dog (1), or total gastrectomy in the dog (2), cat (3), rat (4) and the human (5), the uropepsin rapidly disappears from the urine in from one to three days; and in the human, the uropepsin is significantly reduced or absent when pernicious anemia exists (6). The relationship of an increased uropepsin output with hypernormal gastric secretory activity, on the other hand, has not been established. The only data on this point are from human subjects and the suggestive relationship may be a coincidence. Gottlieb (7) found the 24-hour uropepsin output tended to be highest in subjects who had hypernormal gastric acidities. Recent studies on peptic ulcer patients, who as a group may be regarded as hypernormal gastric secretors, by Block, *et al.* (8) as well as unpublished data of Grossman (9) indicate that the peptic ulcer population may fall into two groups on this point. One in which the uropepsin output is not distinguishably different from that of normal individuals, and a second group characterized by extremely high uropepsin outputs.

The question which needs to be answered is: Does any agent which stimulates gastric secretion likewise augment the uropepsin output? The present study was an attempt to clarify this point using the cat as the experimental animal. Caffeine and histamine are both potent gastric stimulants, the continued administration of which can cause gastric ulcer in the cat. The purpose of the present study was to determine if uropepsin output could be increased in cats given daily injections of caffeine and histamine in a beeswax-mineral oil mixture.

METHOD

Two series of experiments were performed. In the first study, 4 cats were conditioned for five weeks on a daily ration consisting of a uniform mixture of ground Purina Chow, cooked oatmeal and canned sardines. The diet was the only one used throughout this series. The 24-hour urine specimens for 15 days furnished the control data. The cats were then injected once daily for 14 days with 1 cc. of a beeswax-mineral oil mixture containing approximately 150 mgm. caffeine alkaloid and 0.40 mgm. histamine base, as the diphosphate, into the muscles of the limbs in rotation. The daily drug dose contained approximately one-half the amount of caffeine which can be expected to produce ulcers in 50 per cent of the cats in 10 to 14 days (10, 11)

Received for publication July 1, 1948.

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and about one fifth of the minimum dose of histamine which will produce gastric lesions in the cat (12).

The 3 cats which survived the first study were used in the second series of injections 7 weeks later. After control values were obtained for 10 days on the Chow-oatmeal-sardine diet, found by analysis to contain 8.8 per cent protein, the diet was changed to one of ground raw horse meat which contained 19.4 per cent protein and which constituted the sole diet for the remainder of the study. A second control period of 9 days on the meat diet preceded the second series of drug injections. The drug mixture, given subcutaneously for the first 9 days and into the muscle the next 13 days, contained 150 mgm. caffeine alkaloid and 0.45 mgm. histamine base, as the dihydrochloride instead of the diphosphate as used in the first study.

The urine was collected over 24-hour intervals into 5 ml. of 0.5 N HCl and some toluol. Specimens contaminated with feces were discarded. The uropepsin assays were carried out according to the method previously described. The results are expressed in uropepsin units, $\mu\mu$. A unit of uropepsin is defined as that amount of uropepsin, which when present in 1 ml. of urine and added to 5 ml. of 4 per cent beef hemoglobin substrate, pH 1.75-1.80, for one hour at 37°C. will release digestion-products not precipitated by trichloroacetic acid and equivalent in color-producing value to 1 mgm. of tyrosine with Folin's phenol reagent. The uropepsin output is the product of the units present in 1 ml. of urine and the 24-hour urine volume expressed in ml.

RESULTS AND DISCUSSIONS

In the first series, no change whatsoever could be detected in the uropepsin output over 19 control days and 14 test days. The mean control and test output values on each cat are given in table 1. An attempt was made at the end of the first week to intensify the stimulation by doubling the dose, giving it in two injections 8 to 10 hours apart but no evidence of an increase in the uropepsin occurred in any of the cats. Within 3 days, *cat 1* developed typical caffeine convulsions and died, whereupon the initial dosage was resumed for those remaining. *Cat 1* had eaten well and maintained its uropepsin output to the day of death. At autopsy, a chronic ulcer was found in the pyloric region, which measured 2 mm. in diameter after fixation in formalin.

Five weeks later this experiment, with slight modification, was repeated on the 3 surviving cats in order to see if the completely negative results of the first study could be duplicated when another diet, higher in protein content, was employed. A change to a diet rich in meat proteins greatly increases the uropepsin output in the human (2).

During 9 days on the raw meat diet (19.4 per cent protein) all 3 cats significantly increased their uropepsin outputs over that for the 10 days on the Chow-oatmeal-sardine diet (8.8 per cent protein). These data indicate that the organs concerned with uropepsin output in these cats were capable of responding to proper stimulation and that the failure of the drugs to augment the uropepsin output in the first study was not due to organs already working to maximum capacity or in a refractory state.

In the first 9 days of the drug administration, a further increase in uropepsin output occurred in all 3 cats, but in only 2, *cat 2* and *cat 3*, was this increase statistically significant. In the course of the next 13 injections, one of these animals, *cat 2*, became indisposed, refused its food and in 6 days its uropepsin output fell to an average value half the previous level. The animal was killed and examined but no evidence of ulcer was found. *Cat 3* and *cat 4* ate well and maintained a high level of uropepsin output throughout the period of the injections. *Cat 4* was then killed and *cat 3* was followed for 14 days to note the recovery progress. The uropepsin output of this animal returned to the meat diet control level.

TABLE 1. MEAN 24-HOUR UROPEPSIN OUTPUTS OF CATS ON TWO TYPES OF DIET, WITH AND WITHOUT DAILY INJECTIONS OF CAFFEINE-HISTAMINE

	DAYS	CAT 1	CAT 2	CAT 3	CAT 4
		<i>uropepsin units</i>			
<i>Series I</i>					
Control diet (Chow-oatmeal-sardine)	19	46.0	23.5	31.6	39.2
Test I (Control diet + drugs)	14	42.1	26.4	29.1	37.7
<i>Series II</i>					
Control diet (Chow-oatmeal-sardine)	10		24.6±2.1 ¹	13.0±1.1 ¹	23.0±1.8 ¹
Control diet (Raw horse meat)	9		35.8±2.2	34.3±3.4	50.7±8.6
Test II (Meat diet + drugs)	9		54.0±6.4	54.1±5.2	60.3±5.8
Test II (Meat diet + drugs)	12		25.5±1.8	58.0±5.1	58.5±4.2
Control III (Meat diet alone)	14			33.2±3.1	

¹ These are the standard errors of the mean.

On the low protein diet, used in the first series, the caffeine-histamine treatment had no effect on the uropepsin output, although the fact that one cat died in convulsions and had a chronic ulcer would indicate that the drug treatment was potent and that gastric secretion had been stimulated.

In the presence of a high protein intake, the caffeine-histamine treatment conditioned an increased uropepsin output in all 3 cats, but this increase was significant in but 2 cats, and when one of these refused its food, the drugs alone were unable to maintain the uropepsin output. The authors have noted that refusal to eat, in the cat, is always attended by a severe drop in the uropepsin output. Whether the output in *cat 2* was higher in the presence of the drug treatment than would occur in an untreated cat refusing its food cannot be said. Only one of the animals, *cat 3*, showed a significant increase in uropepsin output which could be attributed to the caffeine-histamine stimulation and this increase occurred only when the animal was eating the high protein diet of horse meat. In this instance, discontinuance of the drug in-

jections was attended by a significant fall in the uropepsin output to the previous meat control level.

CONCLUSION

The failure to detect any response when the cats were eating the low protein diet and to find a definite increase in the uropepsin output in all cats, at least initially, when eating a high protein diet indicates that under the conditions related the character of the diet and appetite are more potent factors in altering uropepsin output than caffeine and histamine which are known to have a stimulating action on gastric secretion quite independent of the kind and quantity of food eaten.

While a functioning gastric mucosa is essential for the existence of uropepsin in urine we believe that the amount of uropepsin put out in 24-hours is quite unrelated to the quantity of gastric juice secreted into the stomach, but more evidence is necessary to establish this contention.

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EFFECT OF RENIN ON PROTEINURIA AND PAH CLEARANCE AT LOW PLASMA LEVELS¹

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IN 1940 Pickering and Prinzmetal (1) reported that strongly pressor doses of renin profoundly influenced and modified renal function in the rabbit. This modification was manifested in a marked diuresis, with increased excretion of sodium and chlorides. These changes in renal function were attributed to a direct action of renin on the tubules; the changes persisted as long as renin was given and after tachyphylaxis had abolished the pressor action. Since renin may have a primary function of altering kidney activity and metabolism, we have extended these studies to include other phases of renal function in the rabbit. Particular attention was paid to glomerular damage, by studying proteinuria and determining changes in permeability of the glomerular membrane to injected hemoglobin. Preliminary experiments confirmed the observation that the diuresis resulting from the injection of pressor doses of renin in the rabbit was accompanied by an increased proteinuria (1).

METHODS

All observations included a control period and the period of maximum renin diuresis.

Unanesthetized male rabbits weighing between 1.5 and 3.0 kgm. were used throughout. The animals were kept on their usual diet of commercial rabbit chow up to the evening preceding an experiment when all food was withdrawn and water allowed *ad libitum*. The morning of the experiment, the animal was hydrated with 50 to 100 cc. of warm tap water² by stomach tube. Approximately one hour before the start of a control urine collection period, 500 mgm. of creatinine dissolved in 10 cc. of normal saline was administered subcutaneously. This produced initial blood levels between 10 to 20 mgm. per cent. The animal was then tied to a board and his bladder thoroughly emptied and washed, using a No. 8 F catheter, to insure that subsequent protein determinations did not reflect mucin and detritus from the lower urinary tract. The catheter was cut short to remove as much dead space as possible in the urine collections and was allowed to remain in place throughout the period of observation.

To determine the clearance of para-aminohippuric acid, 250 mgm. PAH³ in 10 cc. saline was given subcutaneously approximately 15 minutes before the expected start

Received for publication May 3, 1948.

¹ This work was carried out with funds provided by the Life Insurance Fund for Medical Research.

² Animals that were to receive hemoglobin were given 0.5 gram of sodium bicarbonate in the water.

³ Supplied by Sharpe and Dohme, Inc. as sodium para-aminohippurate.

of a control urine collection period. This gave initial blood levels between 3.5 to 8.0 mgm. To study hemoglobin permeability, the procedure of Monke and Yuile (2) was used. Initial blood levels of hemoglobin between 250-500 mgm. per cent were obtained by the intravenous injection of 350 to 750 mgm. of rabbit hemoglobin, as 10 to 20 per cent solution, 10 minutes before the start of the control period. Hemoglobinuria was apparent about 7 minutes following the injection.

Urines were collected directly into graduates and midperiod blood specimens obtained by direct cardiac puncture. Particular care was taken to prevent hemolysis.

Following a control period in which specimens for creatinine clearance, PAH clearance, hemoglobin permeability and urinary proteins were collected, a few minutes were allowed to elapse during which time the bladder was again washed and emptied. The animal was then given 10 mgm. of renin⁴ in 2 cc. of physiological saline intravenously. Gross inspection of the rate of urine flow was found to be a satisfactory method of estimating the period of peak diuresis, during which the post-renin observations were made. These peak periods usually occurred about 20 minutes after the renin injection. No animal was used more than once.

Determinations of creatinine in plasma and urine were done by the method of Folin and Wu (3); PAH determinations in plasma and urine by the method of Smith, *et al.* (4). The determination of hemoglobin concentrations in both the hemoglobin solution used for the injection, and standard solutions used for determining the plasma and urine hemoglobin concentrations were done by the iron method of Wong (5). The plasma and urine hemoglobin concentrations were determined by a modification of the cyanmethemoglobin method of Evelyn and Malloy (6). A Klett-Summerson photoelectric colorimeter was used for all color reactions.

In determining proteinuria, the Shevky-Stafford method (7) using Tsuchiya's reagent was used. When hemoglobin was also present in the urine, the volume precipitate for hemoglobin was subtracted from the total using a graph of precipitates from known concentrations of hemoglobin. It was found that weight for weight, both hemoglobin and albumin gave about the same amount of precipitate with Tsuchiya's reagent. In animals made hemoglobinuric, the high control period urine concentration of hemoglobin might account for perhaps nine tenths of the total protein (hemoglobin and albumin) precipitated. The albumin determination therefore was below the limits of desired accuracy, and control period proteinuria is recorded as less than 0.3 mgm. protein/minute. The calculated values were 0.1 to 0.3 mgm./minute. During the period of renin diuresis, the urinary hemoglobin accounts for about one half or less of the total precipitate, and determinations of urinary protein after subtraction of the hemoglobin precipitate is accurate. The validity of these conclusions was borne out by data obtained in animals that were not given hemoglobin, and their urinary protein per minute during a control period and during renin diuresis determined gravimetrically. The gravimetric figures were in concordance with the Shevky-Stafford method of quantitating urinary protein in the presence of hemoglobin, during renin diuresis.

⁴ Hog renin supplied through the courtesy of Dr. K. Kohlstaedt of Eli Lilly Co. A 10 mgm. dose tested in anesthetized animals by carotid artery B.P. gave rises in pressures of 30-40 mm. Hg which reached a peak within 30 seconds and returned to base-line in about 20 minutes.

In all animals a simple qualitative Benedict's test for sugar was done on the control urines and the urine obtained during peak renin diuresis.

Six animals were killed following an experiment and their kidneys removed for microscopic examination.

RESULTS

In carrying out these experiments, a total of 12 animals were used. As had been mentioned, not all procedures were carried out in all animals simultaneously. Most observations were made during a control period and during peak renin diuresis. Of

TABLE I

ANIMAL NO.	A	B	C	D	E	F
	URINE FLOW	CLEARANCE		PROTEINURIA	GLYCOSURIA	HEMOGLOBIN PERMEABILITY
		CREATININE	PAH			
	<i>cc/min.</i>			<i>mgm./min.</i>		
1	0.45	13.5	42.1	0.03	Trace	—
	1.55	9.7	32.5	1.1	Neg.	—
2	—	4.6	28.6	—	—	—
	—	3.8	11.1	—	—	—
3	0.45	12.9	37.4	0.26	one plus	—
	1.92	14.5	18.7	2.6	neg.	—
4	0.14	4.9	36.4	>0.3	one plus	5.0
	1.92	5.2	20.4	1.7	neg.	nd.
5	0.50	10.7	47.9	—	—	—
	2.00	9.4	28.0	—	—	—
6	—	9.0	40.3	—	—	—
	—	6.2	17.9	—	—	—
8	0.20	5.6	28.6	>0.3	one plus	4.8
	2.12	7.6	27.3	2.4	neg.	4.9
9	—	—	—	—	—	4.9
	—	—	—	—	—	nd.
11	0.25	8.0	41.2	>0.3	trace	4.4
	2.10	13.6	40.8	1.7	neg.	4.6
12	0.41	5.9	—	—	—	—
	1.86	7.1	—	—	—	—
X	—	—	—	0.14	—	—
	—	—	—	1.1	—	—
Y	—	—	—	0.22	—	—
	—	—	—	1.4	—	—

the 12 animals, 8 gave a minimum of about four-fold increase in urine flow per minute during renin diuresis over control periods, although there was a definite increase in flow in all animals. Nine sets of observations of the glomerular filtration rates were made. Eight sets of observations of the clearance of PAH were made, using subcutaneous injections of NAPA. Two additional animals were observed for PAH clearance using Forster's (9) intravenous infusion technique. These animals gave very similar results to the subcutaneous injection method, and so intravenous infusion was abandoned since it was felt that this would alter urine flows. A total of five observations of proteinuria were done, and the accuracy checked against observa-

tions in two animals not given hemoglobin. In these, urinary proteins were determined gravimetrically.

In table 1 are recorded the typical observations made on the 12 animals. The upper figure of each pair is the control datum, and the lower is the datum during renin diuresis.

Column B records the creatinine clearances. These clearance figures are a measure of the glomerular filtration rate in the rabbit, as has been pointed out by Kaplan and Smith (8), and correspond with the thiosulphate clearance (10), as we were able to observe in 10 experiments on rabbits.

In *column C* are recorded the clearances of PAH during a control period and during renin diuresis in eight animals. The subcutaneous method gave results similar to

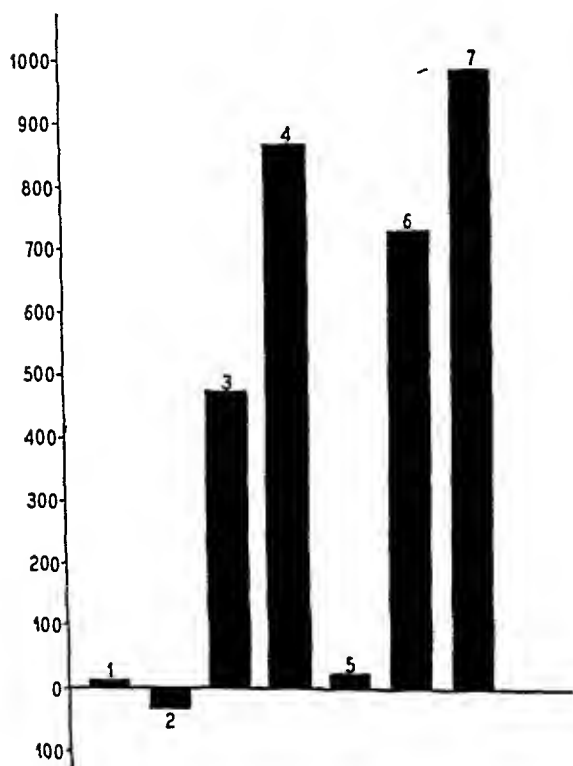


Fig. 1. FUNCTIONAL CHANGES in the kidney during renin diuresis expressed as percentage change from control period; *columns 6 and 7* based on data of Pickering and Prinzmetal (1). 1, glomerular filtration rate; 2, PAH clearance; 3, urine flow/min.; 4, proteinuria/min.; 5, glomerular permeability to hemoglobin; 6, urinary sodium/min.; 7, urinary chloride/min.

those with continuous infusion. Inasmuch as extraction ratios were not done, it is not certain that the clearance figures truly reflect changes in effective renal blood flows. However, the work of others (12) indicates that the effective renal blood flows are diminished with renin, as would be expected with a vasoconstrictor.

Column A records the changes in urine flows during the control periods and during renin diuresis.

Column D lists the quantitative protein determinations. *Animals 1 and 3* received no hemoglobin; *animals 4, 8, and 11* received hemoglobin. These quantitative protein determinations were done by the Shevky-Stafford method. *Animals X and Y* received no hemoglobin and their urinary proteins were determined gravimetrically. In *animal X*, during the decline in diuresis, about 80 minutes after the injection of

renin, when the urine flow had dropped from 3.7 cc./minute at its peak to 0.8 cc./minute, proteinuria also dropped from 1.1 mgm./minute to 0.07 mgm./minute.

Column E records the results of the Benedict's test for sugar in the control period urine, and in the urine during peak renin diuresis.

Column F records the hemoglobin permeability.

DISCUSSION

Addis (13) pointed out that proteinuria is normal in the human and is pathological only when present in excess amounts. Oliver (14) notes the presence of protein molecules in the tubules and tubular cells of what are considered normal mammalian kidneys, using protein-dye combinations.

Methods of studying proteinuria dynamically had never met with great success, until Dock (15) suggested that the hemoglobin molecule might serve the purpose of ruling out changes in the permeability of the glomerular membrane to account for changes in protein excretion, when hemoglobinuria and albuminuria are studied simultaneously. The hemoglobin molecule has 97 per cent the molecular weight of the albumin molecule, and above T_m values, is cleared by the kidney in a definite and predictable proportion of the filtration rate. This proportion expressed in per cent is the ratio of the simultaneous clearance of hemoglobin above T_m values to the glomerular filtration rate and has been designated by Monke and Yuile (2) as the hemoglobin permeability. This figure is interpreted by them to indicate the per cent of pores of the glomerular membrane large enough to allow the passage of an undissociated hemoglobin molecule. This investigation showed (*column F*, table 1) that under normal conditions the glomerular membrane of the rabbit has about 4 to 5 pores per hundred that are large enough to permit the passage of hemoglobin molecules. With these basic facts one can then infer whether proteinuria is the result of a 'damaged' (more permeable) glomerulus or possibly is the result of some other factors acting on or in the kidney.

It is to be understood that although the hemoglobin and the albumin molecules have about the same molecular weight it does not follow that their glomerular permeabilities are alike, since shape and electrical charge may be different. Hemoglobin permeability is about 5 per cent and albumin permeability less than 1 per cent (15, 16).

From the average glomerular filtration rates for the animals examined (8.3 cc. before renin and 8.6 cc. after renin), it is apparent that the filtration rate is unchanged but there is a marked increase in urine flow (average 0.31 cc. before renin and 1.78 cc. after renin). Diuresis is the result of diminished tubular reabsorption of water. The only other explanation for the diuresis lies in the possibility that it is osmotic in nature. That such is not the fact is adequately established by Pickering and Prinzmetal (1). The clearances of PAH (*column C*, table 1) indicate that there is probably a significant fall in the effective renal plasma flow, which, in conjunction with an insignificant change in the glomerular filtration rate, indicates a rise in the filtration fraction and efferent arteriolar constriction. Could the resultant increase in intraglomerular pressure account for the proteinuria? The data in *columns D* and *F* indicate that in spite of this increased pressure the glomerular membrane is no more

permeable to the hemoglobin molecule during renin diuresis. The clearance of hemoglobin varies with the filtration rate and there is no change in ratio of hemoglobin clearance to the creatinine clearance. It seems safe to conclude from this that no greater amounts of protein are appearing in the glomerular filtrate during renin diuresis than during the control period. The proteinuria must therefore be the result of diminished tubular reabsorption of protein. This depression of tubular reabsorption is a purely physiological and transient phenomenon, since, the subsidence of the diuresis is accompanied by a decrease in the amount of protein appearing in the urine. One may indulge in the luxury of calculating the amount of protein in rabbit glomerular filtrate, assuming that the concentration in glomerular filtrate was constant and no albumin was reabsorbed during renin diuresis. Such calculations, based on table 1, give values of 11 to 33 mgm. per cent, with 4 out of 5 animals showing less than 20 mgm. per cent. These figures are concordant with experiments in which the tubules have been paralyzed by cold and a glomerular filtrate obtained (15).

In spite of the general depression of tubular reabsorptive capacity during renin diuresis, glucose does not appear in the urine. It is noted in *column E* that all but one animal had a slight glycosuria during the control period. This glycosuria has been interpreted as probably a psychogenic effect rather than anything pathological. The absence of detectable glycosuria or increase in hemoglobinuria during renin diuresis, when tubular reabsorption of sodium, chlorides, water (1) and protein are depressed, suggest a possible dissociation of tubular activity.

Renin can be isolated from the kidneys of normal animals and produces its pressor effects by catheptic action on plasma protein. It may be that its action on the renal tubule is of far greater biologic importance than its participation in the pressor response to renal ischemia just as the effect of pitressin on the tubule seems more important than its pressor action. By reducing the work of reabsorption at times when renal blood flow is greatly reduced, renin may play a part in the reduction of total renal metabolism observed by Van Slyke and his associates (17). In the rabbit, no rise in arterial pressure was noted after removal of a clamp causing complete renal artery occlusion for two hours; and 23 of 30 animals survived (18).

Microscopic examination of the kidneys of 6 rabbits used in these experiments failed to show evidence of any renal damage.

SUMMARY

1. Strongly pressor doses of renin in the unanesthetized rabbit produce a marked diuresis unaccompanied by alterations in the glomerular filtration rate. As shown by others (1) the diuresis is due to an inhibition of tubular reabsorption of water and is associated with inhibition of reabsorption of sodium and chloride.

2. Strongly pressor doses of renin given to the hydrated unanesthetized rabbit produce a slight decrease in PAH clearance and a marked increase in proteinuria. The increased proteinuria is not accompanied by any significant change in the permeability of the glomerular membrane to injected hemoglobin, and is apparently due to diminished tubular reabsorption of protein.

3. The marked alterations in renal function produced with pressor doses of renin are transient and are not accompanied by any lesions that can be seen microscopically.

The authors express their thanks to Dr. Jean Oliver for reviewing the microscopic sections of the kidneys.

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EXCRETION OF SODIUM AND WATER DURING OSMOTIC DIURESIS IN THE DOG¹

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THE application of the micropuncture technique (1) to single nephrons has shown that most of the filtered sodium chloride and water are reabsorbed in the proximal tubule, and that the tubular urine remains essentially isosmotic with the plasma to a point about half way down the tubule, or to the most distal point available to micropuncture in the intact mammalian kidney. It follows from the isosmotic nature of the tubular urine that water and electrolytes are reabsorbed by the proximal tubule in approximately equal proportions, and thus the osmotic pressure of the reabsorbate is approximately equal to that of the glomerular filtrate and hence to the osmotic pressure of the plasma. It will be recalled that Cushny (2) conceived of the tubular reabsorbate as an 'ideal fluid', containing all essential inorganic constituents in those proportions which characterize normal plasma, and the question therefore arises whether or not the simultaneous reabsorption of electrolytes and water represents the reabsorption of an 'ideal fluid' in Cushny's sense, or merely the coincident operation of two fundamentally independent processes. The experimental work described in this paper was designed to examine this question and demonstrates, we believe, that the latter explanation is the correct one.

When an unreabsorbed solute of low molecular weight (urea, glucose, mannitol, sucrose, sodium thiosulfate, etc.) is introduced into the plasma and hence into the glomerular filtrate, the rate of urine flow is increased roughly in proportion to the plasma concentration of that solute (osmotic diuresis). Shannon (3), using sodium sulfate in the dog, caused the excretion of 57 per cent of the glomerular filtrate at a creatinine U/P ratio of 1.75, and Smith and his co-workers (4) reached a urine flow of 41 cc. per minute and an inulin U/P ratio of 3.5 in man by means of glucose. Evidence has been presented elsewhere (5) that some 85 per cent of the filtered water and sodium chloride are normally reabsorbed in the proximal system, using this term to indicate the proximal tubule and the thin limb of the loop of Henle. Since the greater part of the water of the glomerular filtrate may be excreted during osmotic diuresis, it follows that most, if not all, of this increased excretion must be a result of decreased reabsorption in the proximal system. Under conditions of moderate to extreme diuresis, the contributions of the distal tubule in modifying the composition of the urine are overwhelmed in the flood of proximal diuresis, so that the composition of the urine approaches the composition of the proximal urine, i.e., of the tubular urine as it is delivered from the thin limb to the distal tubule. We have therefore used osmotic diuresis to examine the conditions governing salt and water reabsorption in the proximal system.

METHODS

Trained, unanesthetized dogs were used. Food and water were withdrawn from the animals 15 hours before the experiment. Following three control periods osmotic diuresis was induced by

Received for publication June 1, 1948.

¹ Certain features of this study have been described in a preliminary communication (5).

infusing 25 per cent mannitol solution at a rate between 0.7 and 1.0 cc. per kgm. of body weight per minute. The filtration rate was determined by the exogenous creatinine clearance. Clearance procedures and creatinine determinations were carried out as described by Smith, *et al.* (6). Infusions were given into a vein of the forelimb. Blood samples were withdrawn from an inlying needle in the femoral artery, using 0.1 cc. of Liquaemin (Hoffman-La Roche) per 10 cc. of whole blood as anti-coagulant. The blood was collected with precautions against the loss of CO_2 . Urine was collected through an inlying catheter. Specimens of urine for the determination of pH and CO_2 were collected directly from a urethral catheter into Van Slyke-Cullen pipettes at the midpoint of each urine-collection period. It was usually necessary to terminate the experiment after about three-quarters of an hour of infusion of mannitol solution; a longer period of infusion at the rate and concentration of mannitol used here endangered the life of the animal. Chloride was determined by the method of Sendroy as modified by Van Slyke and Hiller (7), sodium and potassium by flame spectrophotometry², calcium by the method of Roe and Kahn (8), phosphate by the method of Fiske and SubbaRow (9), mannitol by the method of Corcoran and Page (10) and pH with the Cambridge pH meter using a MacInnes glass electrode.

CALCULATIONS

In the formation of the glomerular filtrate, an essentially protein-free fluid is separated from plasma-containing cations and anions in concentration differing from those in the plasma in accordance with the Donnan equilibrium. Because the pH of the plasma is on the alkaline side of the isoelectric point of the plasma proteins, considerable base is bound to plasma proteins and unavailable for filtration, and the concentration of diffusable cations in the ultrafiltrate will be less than that in the plasma water, the concentration of diffusable anions being correspondingly greater (Donnan effect). We have not determined the plasma protein concentration in our experiments but have taken as average values for the Donnan factors corresponding to 6 grams per cent of total protein, $k_{\text{Na}} = 0.925$, $k_{\text{K}} = 0.85$, and k_{Cl} and $k_{\text{HCO}_3} = 1.023$ (11-13). The Donnan factor has been neglected in our calculations dealing with Ca and PO_4 . Assuming that the Donnan factor remains constant at any plasma concentration of a given ion, the concentration of that ion in the glomerular filtrate

(G) is then equal to $k \frac{P}{W}$, where P is the plasma concentration in mEq. per liter and

W the per cent of water in the plasma. In later work, we observed in a typical series of observations that the concentration of plasma protein declined from approximately 6 to 4 grams per cent during the infusion of hypertonic mannitol solutions, and this correction has been applied to all experiments. Assuming that the specific gravity of the protein is 1.0, then 100 minus the protein concentration in grams per cent gives the percentage of filterable water in the plasma, or W as used above.

Recent evidence (10, 14) indicates that some 10 per cent of commercially available mannitol is reabsorbed by the renal tubules. We have neglected this fact in our calculations for the reason that we do not know the extent of tubular reabsorption at the high plasma levels existing in our experiments, nor is it possible for us to make this calculation accurately with reference to the creatinine U/P ratio, since the plasma concentration of mannitol is rising so rapidly and the urine-collection periods are so

² We are indebted to Dr. Nelson Young of the Sloan Kettering Institute and to Dr. Charles Fox, Department of Bacteriology, College of Physicians and Surgeons, Columbia University, for the use of their flame spectrophotometers.

short that the errors in the calculations are relatively large. Accordingly the mannitol concentration in the glomerular filtrate (G_m) has been calculated by dividing the concentration in the urine by the WU/P ratio for creatinine. In order to equate the osmotic pressure of mannitol with that of electrolytes, the mannitol concentration of the plasma and urine has been converted to 'ion equivalents' by conversion to mM/l and divided by 0.93, a factor representing the deviation of the freezing point of 0.1 M NaCl solution from ideality (15). One half of the ion equivalent represents the contribution of the mannitol to the osmotic pressure of the anions and cations, respectively, in the plasma or urine.

RESULTS

Seven experiments were performed in 4 dogs. Pertinent data in two experiments are given in table 1, the results of all other experiments being in general consonant with the two which are reported. These data indicate that during osmotic diuresis *a*) the reabsorption of sodium is an active process; *b*) sodium and water are not reabsorbed in a constant ratio and these two operations are fundamentally independent; *c*) the reabsorption of water (in the proximal tubule) is a passive process; and *d*) the proximal reabsorption of sodium appears to be limited by a critical concentration gradient of this ion between plasma and urine.

That the reabsorption of sodium by the tubular system as a whole involves an active process is evident from the fact that under normal conditions, both at low and at high rates of urine flow, the urine may contain only traces of sodium. Assuming that the proximal tubule is permeable to sodium and water but not to mannitol, the first two might be removed from the tubular urine, with consequent concentration of the mannitol, at the expense of the hydrostatic pressure of the tubular urine. At any reasonable magnitude for this pressure, however, only a small percentage of the sodium and water could be thus removed at plasma mannitol concentrations and urine flows as high as in our experiments. It follows that proximal sodium reabsorption is largely if not wholly an active process, the energy for which is supplied by the tubular cells. This circumstance does not exclude some passive transport of sodium under normal circumstances.

That sodium (and chloride) and water are not reabsorbed in a constant ratio as diuresis increases in magnitude is shown in table 1 and figure 1. In figure 1 the percentage of filtered sodium, chloride, bicarbonate and water excreted in the urine (*dog* 3, exp. 2) are plotted against the log U/P creatinine ratio. As diuresis develops, an increasing fraction of the water of the glomerular filtrate appears in the urine and the log U/P ratio of creatinine decreases from its initial value of 10.4, approaching 1.0 as a limiting value. At the highest urine flow obtained, 31.6 cc. per minute (*dog* 3, exper. 2) 63 per cent of the filtered water was excreted in the urine whereas only 26 per cent of the filtered sodium, 33 per cent of the filtered chloride and 9 per cent of the filtered bicarbonate were excreted. Tubular reabsorption of phosphate remained complete, whereas approximately 95 per cent of the filtered potassium was excreted. Values of the percentage excretion of sodium and water at the maximal urine flows obtained in other experiments are as follows: *dog* 1, exper. 1: sodium, 18.5 per cent, water, 51.5 per cent; *dog* 2, exper. 1: sodium, 13.4 per cent, water, 42 per cent; *dog* 2, exper. 2:

TABLE 1. CLEARANCE DATA FROM TWO OSMOTIC DIURESIS EXPERIMENTS

PERIOD	URINE FLOW	CREATININE U/G RATIO	FILTRA-TION RATE	GLOMERULAR FILTRATE CONCENTRATION ¹					URINE CONCENTRATION					REABSORBATE CONCENTRATION		
				Sodium	Potassium	Chloride	Bicarbonate	Mannitol (cation equiv.)	Sodium	Potassium	Chloride	Bicarbonate	Mannitol (cation equiv.)	Sodium	Chloride	Bicarbonate
	cc/min.		cc/min.	millimols/liter					millimols/liter					millimols/liter		
Dog 3, exper. 2: diuresis induced by infusion of 25% mannitol solution at the rate of 13.4 cc/min. for 50 min. Wt. of dog, 12 kgm.																
Control	4.6	10.4	47.8	140	2.7	123.5	25.9	0	115	17.0	118.8	12.5	0	142	123	27
4	13.2	3.97	52.4	136	2.5	116.0	25.0	11	85	8.4	87.3	5.8	43	153	126	32
5	20.4	2.39	48.6	125	2.6	103.1	23.2	31	69	6.0	71.8	4.2	75	165	126	40
6	22.4	2.18	48.8	121	2.7	102.1	22.0	43	63	5.4	67.1	3.7	93	170	132	38
7	29.0	1.94	56.2	115	2.8	95.9	19.9	58	54	5.0	57.9	2.6	112	181	136	38
8	27.8	1.78	49.4	108	2.8	90.6	18.0	74	49	4.4	52.1	2.5	132	185	140	38
9	31.6	1.60	50.2	103	2.6	88.1	16.6	95	44	4.0	46.7	2.4	151	204	159	41
Dog 2, exper. 1: diuresis induced by the infusion of 20% mannitol solution at the rate of 9 cc/min. for 52 min. Wt. of dog, 15.5 kgm.																
Control	1.1	69.7	79.6	147	3.6	128	25.0	0	103	33	176	0.03	0	148	128	25
4	12.0	7.0	83.7	142	3.7	121	24.0	12	88	5.1	93	0.4	83	151	125	28
5	20.0	3.54	70.7	132	3.8	113	22.5	30	58	3.1	60	1.2	105	161	135	31
6	22.8	2.94	67.0	126	3.8	111	21.6	40	48	2.9	50	1.5	118	166	140	32
7	23.1	2.67	61.5	124	3.7	109	21.4	48	42	2.7	45	1.7	129	174	147	33
8	25.3	2.40	60.6	122	3.4	108	20.9	59	39	2.8	42	2.1	141	183	155	35

¹ See text for methods of calculation.

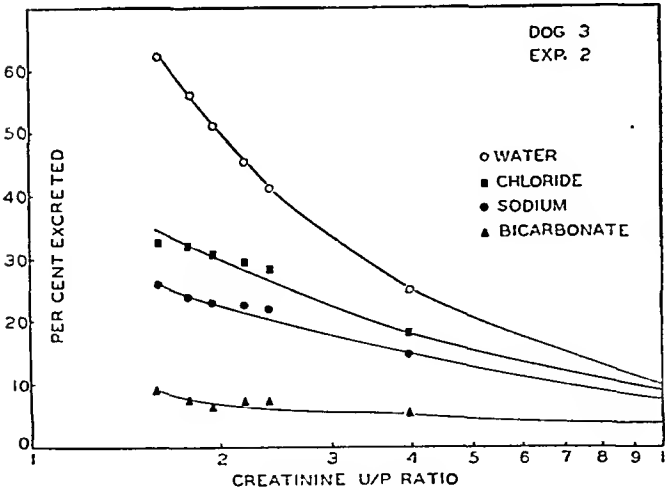


Fig. 1. PERCENTAGE EXCRETION of water, sodium, chloride and bicarbonate in relation to creatinine U/P ratio (log scale) in dog 3, experiment 2. Plotted from data in table 1.

sodium, 17.5 per cent, water, 50 per cent; dog 2, exper. 3: sodium, 9.4 per cent, water, 41 per cent. From these experiments we conclude that the reabsorption of sodium,

chloride and bicarbonate are processes which proceed independently of the reabsorption of water.

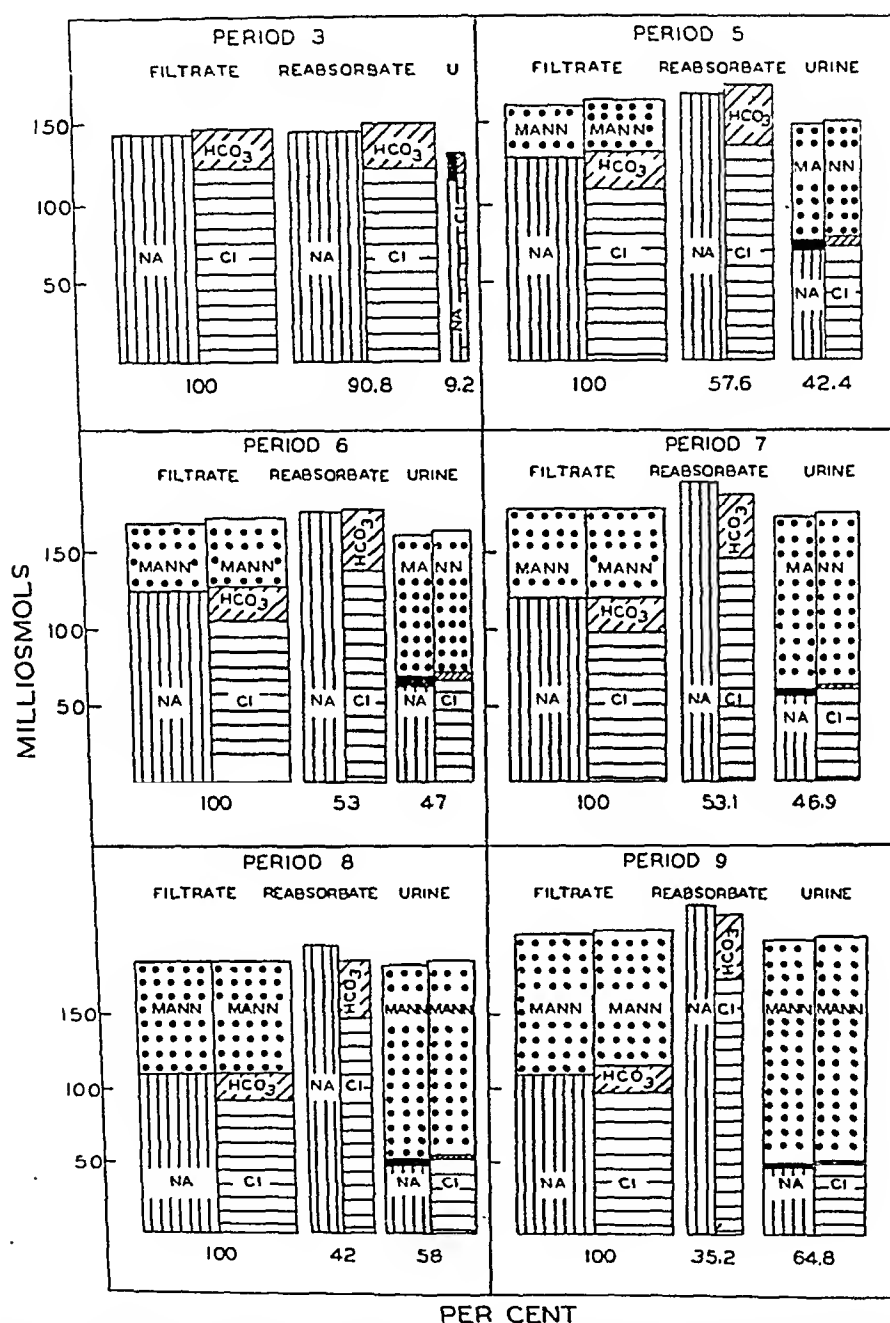


Fig. 2. COMPOSITION IN MILLIOSMOLS of plasma, reabsorbate and urine in dog 3, experiment 2, constructed from data in table 1. The percentage partition of the glomerular filtrate into reabsorbate and urine is represented by the relative width of the blocks.

Figure 2 shows the summated osmolar constituents of the plasma, urine, and of the total reabsorbate during the course of osmotic diuresis in dog 3, experiment 2. Accepting the assumption that the distal tubule only slightly modifies the urine dur-

ing osmotic diuresis, the composition of the total reabsorbate reflects with fair approximation the composition of the reabsorbate in the proximal system. The data show that during osmotic diuresis the osmotic pressure of the tubular urine, and therefore of the reabsorbate, remains nearly identical with that of plasma. This demonstration confirms the conclusion reached by Walker *et al.* (1) from micropuncture studies in the guinea pig and rat.

From the evidence that sodium is reabsorbed actively and independently of water, and that the urine is either isosmotic or slightly hypotonic to the plasma during osmotic diuresis, it is concluded that water returns to the plasma in the proximal tubule by a process of passive diffusion. In this view, water transport will not begin until the active transport of sodium has established an osmotic gradient between the plasma and tubular fluid.

In figure 1 it will be noted that although there is a dissociation between the reabsorption of sodium and the reabsorption of water, nevertheless the rate of excretion of sodium continues to increase with increasing rates of urine flow. Three possibilities for this increased excretion may be considered: *a*) mannitol exerts a toxic action on the tubular cells; *b*) stirring within the tubule may be inadequate to bring all of the sodium to the surface of the tubular cells, making it available for reabsorption. Such a situation might be brought about by an increase in the average velocity of flow of the tubular fluid as an increased volume of urine is discharged down the proximal tubule during the development of osmotic diuresis. *c*) The retention of water in the proximal urine, under the osmotic action of mannitol, may dilute the sodium concentration in the urine below that of the plasma and thus establish increasing concentration gradients between urine and plasma, which progressively reduce the rate of sodium reabsorption.

Since increased sodium excretion also occurs during urea (16) and glucose³ diuresis, (*a*) appears improbable. Since phosphate continues to be completely reabsorbed and bicarbonate almost completely reabsorbed, (*b*) also appears to be improbable. We are then left with the third alternative (*c*) that dilution of sodium in the tubular urine is responsible for decreased reabsorption.

As osmotic diuresis increases in magnitude, the difference in concentration between plasma and urine sodium tends, in any one experiment, to approach a constant value of 60 to 90 milliequivalents per liter which is maintained despite marked variations in the absolute values of the plasma and urine sodium concentration. In addition to direct observation by determination of plasma and urine sodium concentrations, this tendency toward a constancy of concentration difference may be demonstrated by graphical analysis, as follows:

If it is assumed that at high plasma concentrations of mannitol, sodium and mannitol concentrations represent approximately the total osmolar concentration of plasma and urine, and further, that the osmolar concentrations of plasma and urine are equal, as would be the case during isosmotic reabsorption, we may write:

$$1) P_M + P_{Na} = U_M + U_{Na}$$

where P_{Na} and U_{Na} are milliequivalents per liter of sodium in the plasma and urine

³ In experiments not reported here we have observed excretion of sodium during glucose diuresis comparable in magnitude to that observed during mannitol diuresis.

respectively, and P_M and U_M are cation equivalents of mannitol per liter. Assuming no reabsorption of mannitol,

$$2) P_M C_F = U_M V$$

where C_F is the filtration rate and V the urine flow, both in cc. per minute. Substituting U_M in (1), and solving for P_M :

$$3) P_M \left(1 - \frac{C_F}{V}\right) = U_{Na} - P_{Na} \text{ whence } 4) P_M = \frac{V}{C_F - V} (P_{Na} - U_{Na}).$$

Since $C_F - V$ equals T_{H_2O} , the total rate of reabsorption of water in cc. per minute,

$$5) P_M = \frac{V}{T_{H_2O}} (P_{Na} - U_{Na}).$$

In figure 3, P_M from four experiments is plotted against V/T_{H_2O} . The slope of each curve should equal $P_{Na} - U_{Na}$ ⁴. As osmotic diuresis develops, progressively higher values of V/T_{H_2O} are developed and the slope becomes constant, a circumstance

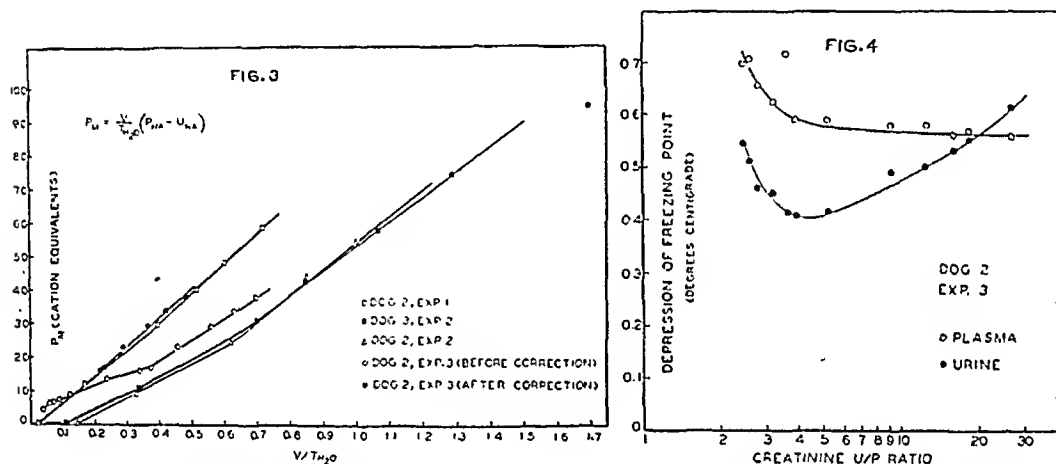


Fig. 3. PLASMA MANNITOL CONCENTRATION, measured as ion equivalents, in relation to V/T_{H_2O} . According to equation 5, the slope of each curve theoretically measures $P_{Na} - U_{Na}$. It will be seen that the slope is constant at high urine flows.

Fig. 4. Depression of the freezing point of plasma and urine during mannitol diuresis, dog 2, experiment 3, in relation to creatinine U/P ratio (log scale).

consonant with the observation that a constant value of $P_{Na} - U_{Na}$ is approached as diuresis increases. Values of $P_{Na} - U_{Na}$ calculated from the slope of the curves in figure 3 are compared with the observed values in table 2, and it will be seen that in general the correspondence is close.

In one experiment (dog 2, exper. 3) the observed value of $P_{Na} - U_{Na}$ was 90 mEq/l. as compared to a calculated value of 61 mEq/l. The large divergence between the observed and calculated values here is probably attributable to failure to achieve isosmotic reabsorption, a condition which was assumed in the derivation of equation 5. Data on the depression of the freezing point of plasma and urine in this experiment (fig. 4) make it clear, however, that isosmotic reabsorption was not ob-

⁴ The significance of the slope of the curves of low urine flows or low values of plasma mannitol concentration is difficult to determine, since the basic assumptions of equation 5 are incompletely fulfilled at these low values. The interpretation of the intercepts of the various curves is also not clear.

tained in this experiment^{5, 6}. The effect which nonisosmoticity of plasma and urine has upon the observed and calculated values of $P_{Na} - U_{Na}$ may be made clear by the following calculation. Using the values for the depression of the freezing point as a basis for correction, we can calculate what V and U_{Na} would have been in each period if the urine had been isosmotic with plasma:

$$6) V' = V \frac{\Delta t_F^u}{\Delta t_F^p}; 7) T'_{H_2O} = C_F - V'; 8) U'_{Na} = U_{Na} \frac{V}{V'}$$

where V' , T'_{H_2O} and U'_{Na} are the values of V , T_{H_2O} and U_{Na} , corrected to isosmoticity,

TABLE 2. OBSERVED AND CALCULATED VALUES OF PLASMA SODIUM CONCENTRATION MINUS URINE SODIUM CONCENTRATION AT HIGH URINE FLOWS IN FOUR EXPERIMENTS

OBSERVED						CALCULATED ¹	OBSERVED					CALCULATED ¹		
Dog No.	Exper. No.	Period No.	Plasma sodium	Urine sodium	Diff.		Dog No.	Exper. No.	Period No.	Plasma sodium	Urine sodium		Diff.	
3	2	millimols/liter				60	73	2	3	millimols/liter			61	
		5	126	69	57					9	123	31		92
		6	122	63	59					10	120	30		90
		7	117	54	63					11	118	28		90
		8	109	48	61					12	115	26		89
		9	104	44	60					Av.				90
		Av.								Av.				90
		Av.								Av.				90
		Av.								Av.				90
		Av.								Av.				90
2	1	5	135	58	77	90	2	3	9	123	43 ²	80	80	
		6	128	48	80				10	120	41 ²	79		
		7	124	42	82				11	118	37 ²	81		
		8	122	39	83				12	115	33 ²	82		
		Av.							81	Av.				81
		Av.							81	Av.				81
		Av.							81	Av.				81
		Av.							81	Av.				81
		Av.							81	Av.				81
		Av.							81	Av.				81
2	2	3	136	64	72	76								
		4	129	56	73									
		5	126	50	76									
		6	124	42	82									
		Av.							76	Av.			76	
		Av.							76	Av.			76	

DISCUSSION

From the data presented here and those which have been reviewed elsewhere (5), the following conclusions may be drawn:

Sodium is actively reabsorbed by the proximal tubule. Concurrently and probably in consequence of the slight osmotic gradient thereby produced, water is passively reabsorbed, tending to maintain the urine in the proximal system isosmotic with the plasma. Since only one eighth of the water of the glomerular filtrate is available for urine formation in water diuresis or diabetes insipidus, it is inferred that this fraction is normally reabsorbed by the distal tubule, and that seven eighths of the water and therefore seven-eighths of the filtered sodium are reabsorbed proximally. It has elsewhere (17) been suggested that the probable function of the thin limb of the loop of Henle is to promote osmotic equilibration between tubular urine and plasma, before the urine is delivered to the distal tubule where the final and critical operations of sodium and water reabsorption are effected.

The proximal reabsorption of sodium appears to be retarded by the development of a critical concentration gradient between tubular urine and plasma, and this critical gradient is reached during mannitol diuresis when the presence of mannitol in the tubular urine prevents the osmotic reabsorption of water and thus dilutes the sodium of the urine. Progressive failure of sodium reabsorption leaves increasing quantities of sodium in the urine which, by its own osmotic pressure, still further prevents reabsorption of water. Hence, failure of water reabsorption in osmotic diuresis is attributable both to the osmotic action of the diuretic (mannitol) and of the unreabsorbed sodium.

As the glomerular filtrate enters the proximal tubule from Bowman's capsule, it has essentially the same concentration of sodium as the plasma, and sodium reabsorption is consequently rapid. Under normal conditions it may be supposed that water reabsorption proceeds about as rapidly as sodium reabsorption so that at no time does the concentration of sodium in the tubular urine fall much below that in the plasma. When a nonreabsorbable solute, such as mannitol, is present, water retention dilutes sodium in the tubular urine, and when the difference between the sodium concentration in the plasma and urine reaches a value of about 60 to 90 mEq/l., reabsorption of sodium is arrested.

The limiting effect of a concentration gradient upon proximal sodium reabsorption is in sharp contrast to the high concentration gradient which may be established between plasma and bladder urine under normal conditions of urine formation, when the sodium concentration of the urine may fall very close to zero. These observations are reconciled by the supposition that sodium reabsorption occurs in two stages: In the proximal tubule, large quantities of sodium are reabsorbed over a small concentration gradient, a circumstance which permits the proximal tubule to reduce the gross bulk of the glomerular filtrate and consequently to concentrate waste products destined for excretion. Distally, a second type of transfer mechanism reabsorbs small quantities of sodium over a much larger concentration gradient. This distal mechanism would thus appear to be similar to the mechanism responsible for the reabsorption of glucose. The fact that it is located in the distal tubule, to which only

a small fraction of the glomerular filtrate is delivered, enables it to effect fine adjustments on a reduced volume of fluid⁷.

SUMMARY

1. Water and electrolyte excretion have been studied during osmotic diuresis induced by infusion of concentrated mannitol solution into dogs.

2. With increasing diuresis, relatively more water than sodium is excreted in the urine. It is concluded that water and sodium are not necessarily reabsorbed in a fixed ratio, and that the simultaneous reabsorption of large quantities of water is not essential to the reabsorption of sodium. It is concluded further that the reabsorption of the bulk of the filtered sodium requires the expenditure of energy by the tubular cells as an active transfer process.

3. Comparison of the concentration of osmotically active solutes in plasma and urine at high urine flows induced by osmotic diuresis shows that the reabsorbate is approximately isosmotic. It is concluded that associated with the active reabsorption in the proximal system of approximately 85 per cent of the filtered sodium, an osmotically equivalent quantity of water is reabsorbed by passive diffusion.

4. The plasma-urine sodium concentration difference tends to attain a constant value at creatinine U/P ratios below 3.5, indicating that this concentration difference is a major factor in limiting the proximal reabsorption of sodium.

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⁷ It is possible that our interpretation of the action of mannitol in retarding the reabsorption of sodium is applicable to unreabsorbed solutes normally present in the glomerular filtrate. Urea, the most important of these, may, by the osmotic pressure developed as it becomes progressively concentrated in its passage down the proximal tubule, restrict the proximal reabsorption of water and thus restrict the proximal reabsorption of sodium. It may be that this action of urea contributes to the circumstance that only 85 per cent of the filtered sodium (and water) are normally reabsorbed in the proximal system.

INFLUENCE OF GASEOUS TRANSFER BETWEEN THE COLON AND BLOOD STREAM ON PERCENTAGE GAS COMPOSITIONS OF INTESTINAL FLATUS IN MAN¹

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THE behavior of gases in the gastro-intestinal tract has been the subject of several studies, and it is generally concluded that the atmospheric air entering the stomach during swallowing and the gases arising in the intestinal tract by fermentation are modified by interchange with the gases of the blood stream. This exchange of gas between the lumen of the intestine and the blood circulating in its wall is expected to be the same response occurring between gases contained in any body cavity and the blood stream. Campbell (1), Henderson (2) and SeEVERS (3) are in agreement that any gas introduced into the pleural cavity, the peritoneal cavity, the bladder or subcutaneous tissues approaches equilibrium with the partial pressures of that gas in the venous blood and tissue fluids. These investigators found equilibrium values for oxygen ranging from about 20 to 30 mm. Hg (2.63% to 3.95%) and for carbon dioxide averaging 50 mm. Hg (6.58%).

In view of the supposition that the behavior of gases in the gastro-intestinal tract is the same as in any body cavity, with the possible exception of the influence of gases produced by bacteria in the colon, the present investigation was undertaken for a more satisfactory explanation of some obvious discrepancies reported pertaining to the normal gas compositions of the intestinal tract.

In an analysis of gas as discharged via the anus, Fries (4) found the following percentage volume compositions: 10.3% carbon dioxide, 0.7% oxygen, 29.6% methane and 59.4% nitrogen. In the case of the stomach, Planer (5) reported 23 to 33% carbon dioxide, 0.8 to 6.1% oxygen and 66 to 68% nitrogen in the dog, while Kantor (6) determined the gases in man as 4.16% carbon dioxide, 17.08% oxygen and 78.82% nitrogen. From his analytical data Ruge (7) concluded that when food is present in the alimentary canal, the oxygen tension is practically nil while carbon dioxide may exist at very high tensions (50% of one atmosphere) in the large intestine. Hibbard (8) studied the gas which forms above obstructions in the small bowel and found that it consisted generally of about 70% nitrogen, 6 to 9% carbon dioxide (a concentration approaching that in the blood) and 10 to 12% oxygen.

McIver and coworkers (9) have stressed the importance of the physical process of diffusion as being the determining factor governing the rate of gas absorptions. They found that the quantity of a given gas which will diffuse across a membrane in unit time depends directly upon the differences in partial pressure of the gas on either side of the membrane, the extent of its surface, and inversely upon its thickness. The absolute value of the rate of diffusion when these conditions are defined is determined by the characteristic of the gas and by the nature of the membrane through which it

Received for publication January 26, 1948.

¹ Presented in part before the American Physiological Society. Federation Proc. 6: 180, 1947.

² Abridgment of portion of thesis submitted to the faculty of the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Ph.D. in Physiology.

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is diffusing. These investigators derived equations predicting the absorption coefficients of various gases, on the basis of the observation that an equilibrium is reached when the partial pressure of the gas in the lumen is equal to the mean tension of the gas dissolved in the circulating blood. This condition of equilibrium should be the same, regardless of the direction from which it is approached. It is interesting to note that in every case they found that carbon dioxide diffuses rapidly, oxygen more slowly and nitrogen hardly at all.

In the experimental work that follows, comparison is made between the absorption coefficient of gases determined on the basis of McIver's equation and the absorption coefficients calculated on the basis of the experimental data reported.

EXPERIMENTAL

The experimental work involved three parts: *a*) the absorption of oxygen as contained in room air, *b*) the absorption of carbon dioxide as contained in a mixture of 25 per cent carbon dioxide and 75 per cent nitrogen, and *c*) the absorption of nitrogen. The behavior of these gases was studied by introducing 500-cc. volumes into the colons of normal healthy male adult subjects by way of an open-tipped rubber tube inserted into the rectum for a distance of about 10 cm. This type of experimentation was always done on individuals who had recently defecated. The subjects were instructed to rest comfortably in a hospital bed in any position desired, since position was not found to influence the results. Gas samples of 30 cc., sufficient for duplicate

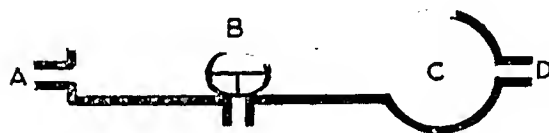


Fig. 1. DIAGRAM OF APPARATUS for collecting alveolar gas samples in equilibrium with gas tensions of venous blood.

analyses, were withdrawn into a 50-cc. syringe at periodic intervals of time, the gas being stored over saturated sodium chloride solution contained in a series of burettes. The gas samples were analyzed for oxygen, carbon dioxide, methane and hydrogen. Nitrogen was considered for all practical purposes as the residual gas and was estimated by difference. The analyses were carried out in an apparatus designed for rapid manipulation and utilizing the usual principles of analysis, oxygen being absorbed by alkaline pyrogallol, carbon dioxide by concentrated potassium hydroxide and methane and hydrogen determined by slow combustion.

Gas tensions in mixed venous blood were determined by a breathing technique, which is a composite of the Plesch procedure as described by Peters and Van Slyke (10), the Henderson and Prince method (11) and the method of Burwell and Robinson (12). The method used was designed to eliminate some of the causes for criticism and include the advantages for each of the techniques. The procedure was carried out as follows:

A rubber bag (fig. 1, *c*) of about 1500 cc. capacity was filled with about 1000 cc. of nitrogen. The nose of the subject was closed with a clip and he applied his mouth to the breathing tube at *A*, the stopcock *B* being turned so that he was connected with the outside air. The subject breathed normally for a short period, after which he emptied his lungs as deeply as possible by a forced expiration into the out-

side air and then quickly inhaled the entire content of the bag by adjusting the three-way stopcock. He then rebreathed deeply the mixture in the bag for 25 seconds, five respirations usually sufficing. At the end of the last expiration into the bag, the stopcock was again turned, shutting off the bag, and the subject removed his mouth from the breathing tube and breathed outside air. The procedure was repeated a sufficient number of times (with 3-minute intervals to allow for return of normal conditions of respiration and circulation) until constancy of composition was found in successive analyses. The gas samples for the analyses were removed at *D*. When the subject was at rest, constancy of composition was attained usually after about the fifth inhalation and exhalation procedure. Thus gas tensions in mixed venous blood were determined indirectly by analyzing carbon dioxide and oxygen tensions in alveolar air which has been retained in the lungs until equilibrated with those tensions in the mixed venous blood coming from the right ventricle of the heart. A more direct method would obviously be to analyze blood from a superficial vein, but

TABLE 1. ABSORPTION OF OXYGEN AS CONTAINED IN AIR

SAMPLE	P OBSERVED	t	P CALCULATED	Δ DEV.	Δ ² DEV.
	%	min.	%		
1	20.93	0	20.59	-0.34	-0.12
2	17.98	10	18.03	+0.05	0.00
3	14.78	25	14.93	+0.15	+0.02
4	12.00	40	12.48	+0.48	+0.23
5	9.87	65	9.46	-0.41	-0.17
6	7.25	95	7.10	-0.15	-0.02
7	5.39	125	5.61	+0.22	+0.05
8	4.72	155	4.68	-0.04	0.00
9	3.39	185	3.10	-0.29	-0.08
Σ.....				-0.55	-0.09

$$\text{Equation: } P \text{ calc.} = 17.43 e^{-0.01553 t} + 3.11$$

this would be impractical for human subjects, while the method described is sufficiently approximate to direct blood analysis to warrant its more practical usage. The described technique is not to be confused with the more commonly used method of determining carbon dioxide and oxygen tensions of quickly expelled alveolar air in equilibrium with the arterial blood.

RESULTS

Absorption of oxygen. According to the diffusion theory, the rate at which a gas flows across a membrane is directly proportional to the difference in pressures on the two sides of the membrane, so that when oxygen, as contained in room air, is introduced into the colon, a pressure gradient exists between that oxygen in the colon and the oxygen tension in venous blood. Therefore, absorption of the oxygen will take place into the blood stream until this gradient approaches zero.

A typical set of data is shown in table 1, demonstrating a progressive decrease in the percentage of oxygen of one atmosphere during the designated intervals of

time, while figure 2 pictures a graph of these results in which the changes in composition of the gas samples were plotted as points describing a curve with the percentage of gas on the vertical axis against time on the horizontal axis.

The type of progressive absorption observed here appears to follow an exponential curve, and therefore, any equation to fit such a curve would have to be of the type $P = ae^{-kt} + c$, where P is the pressure in percentage of one atmosphere, a is the effective initial pressure gradient, e indicates natural logs, k is the absorption coefficient, t is time in minutes and c is a constant determining the limits of the curve and is assumed to be the gas tension in the venous blood. The equation, as calculated on the basis of that specific set of data shown in table 1 and figure 2, is observed to be $P = 17.43 e^{-.01553t} + 3.11$, while the closeness of fit is indicated by a comparison of the observed P with calculated P values.

In figure 2 it is observed that the percentage of oxygen approaches and reaches equilibrium with the venous oxygen tension in a period of 185 minutes. The graph

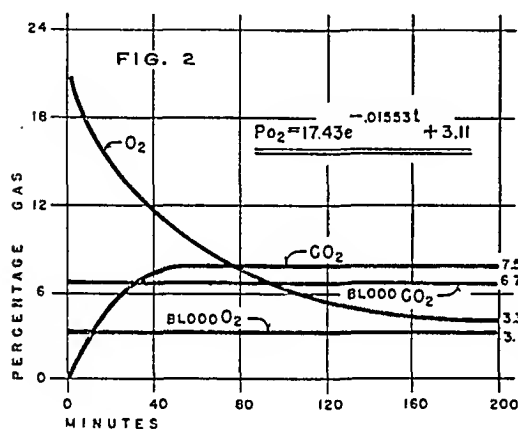


Fig. 2. ABSORPTION OF OXYGEN as contained in air, showing the inward diffusion of carbon dioxide into the lumen of the colon.

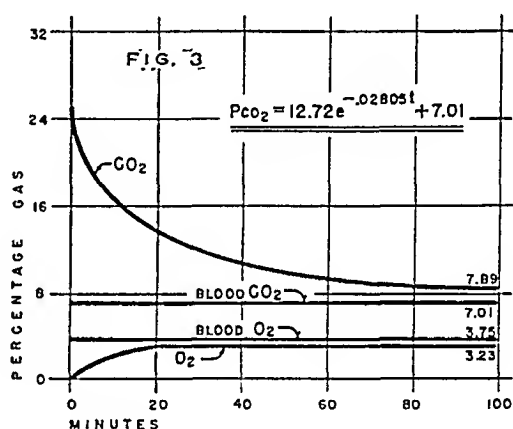


Fig. 3. ABSORPTION OF CARBON DIOXIDE as contained in a carbon dioxide-nitrogen mixture, showing the inward diffusion of oxygen into the lumen of the colon.

also shows the counterprocess of diffusion, that of entrance of carbon dioxide into the lumen of the colon from the blood stream, and indicates the relatively short time in which the tension of carbon dioxide in the colon reaches the venous tension of 6.73 per cent in this specific experiment, as indicated by the straight solid line. The higher tension of carbon dioxide in the colon can logically be accounted for by that amount produced by the intestinal flora.

Table 2 summarizes the results of ten experiments, indicating the equations for the absorption curves, the absorption coefficients as the k value in these equations, the statistical significance of the data, and a comparison of these k values and those calculated by McIver on the basis of an equation derived on theoretical considerations already mentioned. His equation for calculating the k value is as follows: $k = \frac{1}{t} [-\log e (a - x)]$, where $(a - x)$ is the pressure gradient.

The average equation is $P = 17.13 e^{-.01658t} + 3.52$, while the average k value

is $-.01658 \pm .0006$, this average comparing favorably with the average k value of $-.01638$ calculated from McIver's equation. The statistical analyses show a high degree of significance for the results.

Absorption of carbon dioxide. Table 3 and figure 3 indicate the very rapid absorption of carbon dioxide, the greatest volume of the gas being absorbed in the first twenty minutes and attaining a final equilibrium of 7.89 per cent in ninety minutes in this particular experiment. This equilibrium value compares favorably

TABLE 2. SUMMARY OF RESULTS ON ABSORPTION OF O₂ IN THE COLON

EXP. NO.	EQUATION	$-k$ VALUE \pm SE	PROBABILITY	MCIVER'S $-k$ VALUE
1	$P = 17.38e^{-.01971t} + 3.67$	$.01971 \pm .00017$.01	.01838
2	$P = 19.41e^{-.01280t} + 4.25$	$.01280 \pm .00007$.001	.01309
3	$P = 14.05e^{-.01632t} + 3.22$	$.01632 \pm .0001$.01	.01554
4	$P = 13.94e^{-.01856t} + 4.13$	$.01858 \pm .00015$.0001	.01881
5	$P = 17.43e^{-.01553t} + 3.11$	$.01553 \pm .001$.0001	.01557
6	$P = 17.58e^{-.01766t} + 3.46$	$.01766 \pm .00014$.0001	.01683
7	$P = 17.64e^{-.01611t} + 3.40$	$.01611 \pm .0001$.0001	.01685
8	$P = 18.30e^{-.01667t} + 3.33$	$.01667 \pm .0001$.0001	.01639
9	$P = 17.64e^{-.01669t} + 3.21$	$.01669 \pm .0001$.0001	.01643
10	$P = 17.93e^{-.01573t} + 3.41$	$.01573 \pm .0001$.0001	.01591
	$P = 17.13e^{-.01656t} + 3.52$	$.01658 \pm .0006$.01638

TABLE 3. ABSORPTION OF CARBON DIOXIDE IN THE COLON

SAMPLE	P OBSERVED	t	P CALCULATED	Δ DEV.
	%	min.	%	
1	25.89	0	19.73	-6.16
2	20.00	5	18.07	-1.93
3	15.91	10	16.62	+0.71
4	12.56	20	14.27	+1.71
5	10.60	35	11.78	+1.18
6	9.68	50	10.14	+0.46
7	8.64	70	8.79	+0.15
8	8.26	90	8.03	-0.23
9	7.89	95	7.90	+0.01
Σ				-4.10

$$\text{Equation: } P \text{ calc.} = 12.72e^{-.02805t} + 7.01$$

with the venous blood carbon dioxide tension of 7.01 per cent. The equation describing the curve for this experiment is $P = 32.72e^{-.02805t} + 7.01$. It will be observed from the calculated pressure percentages of carbon dioxide that the derived equation fits the curve of absorption less accurately than for the absorption of oxygen. Figure 2 also shows the inward diffusion of oxygen from the blood stream, and again is seen to approach the venous blood tension of 3.75 per cent, the actual value being somewhat lower than this venous tension for the same reason that carbon dioxide remains

at equilibrium above the venous tension, namely, the utilization of oxygen and the production of carbon dioxide by the metabolic processes of the intestinal flora.

Table 4 summarizes the results of ten experiments, indicating the equations for the absorption curves, the absorption coefficients, the statistical significance of these data and a comparison of the k values and those calculated by McIver's equation.

The average equation is $P = 13.89 e^{-.02884 t} + 6.86$, while the average k value is $-.02884 \pm .0006$, this value comparing less favorably with the average k value

TABLE 4. SUMMARY OF RESULTS ON ABSORPTION OF CO_2 IN THE COLON

EXPT. NO.	EQUATION	$-k$ VALUE \pm SE	PROBABILITY	MC IVER'S $-k$ VALUE
1	$P = 9.70e^{-.02577t} + 8.05$	$.02577 \pm .0003$.01	.02772
2	$P = 15.89e^{-.02945t} + 6.39$	$.02945 \pm .0004$.001	.03143
3	$P = 12.72e^{-.02805t} + 7.01$	$.02805 \pm .0003$.001	.03094
4	$P = 15.22e^{-.02904t} + 6.92$	$.02904 \pm .0004$.001	.03129
5	$P = 13.35e^{-.03000t} + 7.03$	$.03000 \pm .0004$.001	.03074
6	$P = 14.51e^{-.02894t} + 6.25$	$.02894 \pm .0004$.001	.03154
7	$P = 14.34e^{-.02892t} + 6.89$	$.02892 \pm .0004$.001	.03113
8	$P = 13.41e^{-.02959t} + 7.42$	$.02959 \pm .0004$.001	.03102
9	$P = 15.53e^{-.02641t} + 6.05$	$.02641 \pm .0014$.05	.02371
10	$P = 14.26e^{-.03221t} + 6.56$	$.03221 \pm .0045$.0001	.03446
	$P = 13.89e^{-.02884t} + 6.86$	$.02884 \pm .0006$.03040

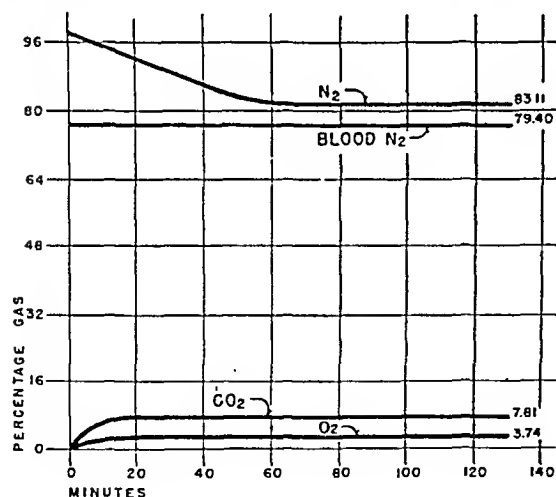


Fig. 4. ABSORPTION OF NITROGEN in the colon, showing the inward diffusions of both carbon dioxide and oxygen.

of $-.03040$ calculated from McIver's equation, presumably because the constant production of carbon dioxide by the flora would act as an artefact in producing an absorption coefficient lower than any theoretical value.

Absorption of nitrogen. By way of demonstrating the low absorbability of nitrogen, figure 4 shows the effect of introducing this gas into the colon. It is observed that very little change in composition occurs, simply approaching the blood tension of the gas, which in this experiment is shown to be 79.40 per cent, no further absorp-

tion taking place beyond this value. The graph also shows the inward diffusions of both oxygen and carbon dioxide into the intestinal lumen from the blood stream until they reach their respective venous blood concentrations.

DISCUSSION

Throughout this work, the importance of the venous blood gas tensions has been stressed in limiting the extent of absorption of gases from the colon. Oxygen, carbon dioxide and nitrogen transfer between the colon and blood stream occur at a rate commensurate with physical principles described by McIver. This absorption rate of gas has been found to depend on the partial pressure gradient existing between the lumen of the colon and the venous blood. The results imply that the percentage volumes of gas compositions in intestinal flatus as discharged via the anus approximate the tensions of these gases in venous blood once an equilibrium has been established, with possible variations due to the presence of different types of intestinal flora. As one would expect, the predominant gas in such flatus is nitrogen. Once nitrogen has gained entrance into the intestinal tract, either from swallowed air or by diffusion from the blood stream, it is not significantly reabsorbed, not only because of its low diffusion constant and low solubility in blood, but also because the blood and tissues are already saturated with this gas existing there under a partial pressure of about four-fifths of an atmosphere and because this entire amount is held only in the form of a physical solution in the blood plasma. This is not the case with oxygen and carbon dioxide, which exist only to a small degree in physical solution, but are present almost wholly in chemical combination in the blood. In connection with this, the very rapid absorption of carbon dioxide during the first 20-minute period, as seen in figure 3, is interpreted as not being strictly a diffusion process but a chemical effect; that is, the formation of bicarbonate in the blood catalyzed by the enzyme carbonic anhydrase. This could well account for the rather poor fit of the exponential equation to the experimental data.

On the basis of averages obtained from numerous analyses of normal flatus, as expelled by way of the anus, and the experimental data already reported, such gas discharges are composed of 3.0% oxygen, 7.5% carbon dioxide and 80.0% nitrogen, the remaining 9.5% being divided between methane, hydrogen and other residual gases. These figures take into account the influence of the intestinal flora and indicate the deviation that could occur from the average venous gas tensions of 3.52% oxygen, 6.86% carbon dioxide and 80.0% nitrogen.

In the light of the experimental results obtained, the lack of uniformity in agreement between previous investigators on the analyses of gastro-intestinal gases may be due, in part, to the interval of time elapsing between the introduction of the gas studied and the final analysis. It would appear that Fries' analysis of methane is considerably higher than one would expect, while Planer's analysis of carbon dioxide is in great excess of what would be expected on the basis of experiments reported here, owing to the rapid diffusion rate of this gas. Both of these high percentages would produce a nitrogen value somewhat lower than might be anticipated, since such nitrogen percentages are calculated by difference.

SUMMARY

The absorption rate of gas in the colon of man depends on the partial pressure gradient existing between the lumen and the venous blood. The average venous blood tension of oxygen was 3.52 volume per cent (27 mm. Hg), proving to be the limiting factor in the extent of absorption of this gas from the colon. The same has been found to be true for carbon dioxide in which the average venous tension of 6.86 volume per cent (52 mm. Hg) limits the extent of absorption for this gas. The high blood tension of nitrogen of about 80.0 volume per cent (608 mm. Hg) retards the absorption of this gas from the colon, thus explaining the predominance of this gas in intestinal flatus.

The authors are indebted to Dr. Kenneth L. Blaxter for advice on the statistical handling of the data.

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EFFECT OF NORMAL BLOOD SERUM AND BLOOD SERUM FROM NEOPLASTIC DISEASE ON CELL PROLIFERATION IN BONE MARROW CULTURES

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BLOOD serum and tissue extracts have been used by many investigators to furnish required nutrients in cell culture experiments. For the culture of bone marrow cells Osgood and Brownlee (1) suggest sterile serum or other special nutrient material. Hays (2), in measuring the reticulocyte increase in bone marrow cultures, found that normal human and rat blood serum gave an increase in reticulocytes, while a single sample of serum from a patient having pernicious anemia in relapse did not. Armburst and Bett (3) used a sheep serum media in studying the effect of liver extracts on the migration of bone marrow cells in explants. Norris and Majnarich (4, 5) found that normal blood serum increased the rate of cell proliferation in bone marrow and tissue cell cultures *in vitro*.

A further study was made of the effect of blood sera upon cell proliferation of bone marrow cultures *in vitro*. A comparison was made between normal blood serum and the blood serum of individuals and experimental animals with neoplastic disease and other pathological conditions.

In the present work beef bone marrow was used and was cultured according to the procedure previously outlined (4, 5). Ten mgm. of casein acid hydrolysate and 0.5 mgm. of tryptophane were added per ml. of bone marrow suspension. Supplements were added as indicated. The final volume in each vial of an experiment was the same. One-tenth ml. of normal blood serum added to 2 ml. of bone marrow suspension increased the rate of cell proliferation approximately equivalent to the increase caused by 5 γ of xanthopterin per ml. of bone marrow suspension. Normal human blood sera obtained from a blood bank fell within a relatively narrow range of response. The response was proportional to the amount of blood serum used in the test as is shown in following experiments.

Figures 1 to 4 show representative results obtained with normal and pathological human blood sera. The responses to xanthopterin and antixanthopterin are also included among the curves to furnish standards for comparison of response.

Figure 4 gives a comparison of response to sera from cases of active tuberculosis and of bronchiogenic carcinoma. The serum from cases of active tuberculosis gave an increase in the rate of cell proliferation, while that from the cases of carcinoma inhibited proliferation to an extent equivalent to 5 γ of antixanthopterin per ml. of bone marrow suspension.

In those conditions in which there was abnormal cell formation, or a neoplastic growth as in pernicious anemia, leukemia and neoplastic disease, there was a very marked change in the response of bone marrow cultures to the blood serum, in that cell proliferation was inhibited.

Curves J and K of figure 3 give the response in 2 pernicious anemia cases. All sera from pernicious anemia yet studied have shown approximately the same range of inhibition of cell proliferation.

Curve J of figure 2 represents serum from myelogenous leukemia; curve D of figure 2 from chronic lymphogenous leukemia; and curve H of figure 1, from chronic myelogenous leukemia. Sera from a number of other leukemias have been tested including an acute lymphatic leukemia, subleukemic type. All leukemias have shown a strong inhibitory effect on bone marrow and tissue cells in *in vitro* culture.

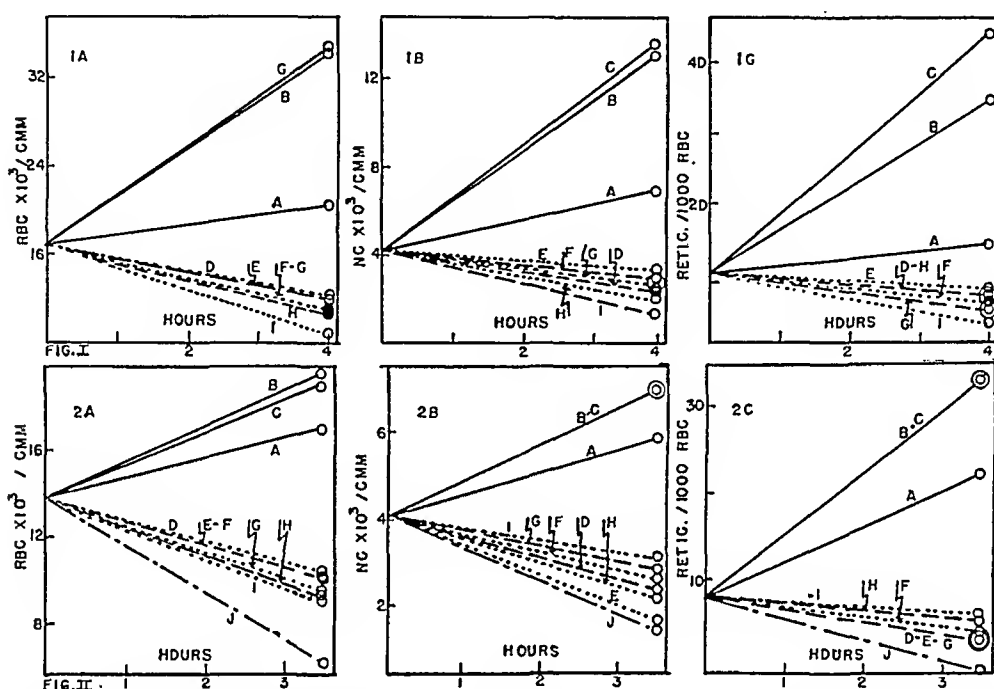


Fig. 1. EFFECT OF PTERIDINES and blood serum on the rate of cell proliferation of bone marrow cells *in vitro*. The following supplements were added including 0.1 ml. of blood serum from the conditions given: A, no supplement; B, 5 γ of xanthopterin per ml.; C, normal blood serum; D, cancer of the lung; E, aplastic anemia; F, papillary carcinoma of the bladder, grade III; G, chronic myelogenous leukemia; H, adenocarcinoma of the prostate; I, carcinoma of the kidney.

Fig. 2. EFFECT OF PTERIDINES and blood serum on the rate of cell proliferation of bone marrow cells *in vitro*. The following supplements were added, including 0.1 ml. of blood serum of the conditions given: A, no supplement; B, 5 γ of xanthopterin per ml.; C, normal blood serum; D, chronic lymphogenous leukemia; E, adenocarcinoma of the prostate; F, papillary carcinoma of the bladder, grade I; G, aplastic adenocarcinoma of the pancreas; H, cancer of the lung; I, adenocarcinoma of the prostate with metastases; J, myelogenous leukemia.

A series of seven sera from human pregnancies gave a uniform response very close to that obtained in the controls with no supplement.

Xanthopterin counteracts the inhibitory effect of serum from cases of neoplasm on the cell proliferation of bone marrow or normal tissue cell cultures. The response to xanthopterin in the presence of blood serum from neoplastic disease was proportional to the concentration of xanthopterin in the same manner as xanthopterin counteracts an antixanthopterin.

A study was made of the response of bone marrow cultures to the serum of

rabbits inoculated with Brown-Pearce tumor. The tumor cells were inoculated into the muscle of the hind leg of each of 6 rabbits. Blood was drawn at intervals by cardiac puncture.

There was a rapid change in the response of bone marrow cells *in vitro* to the blood serum of rabbits after inoculation with Brown-Pearce tumor as shown in figure 5. The normal rabbit serum produced an increase in the rate of cell prolifera-

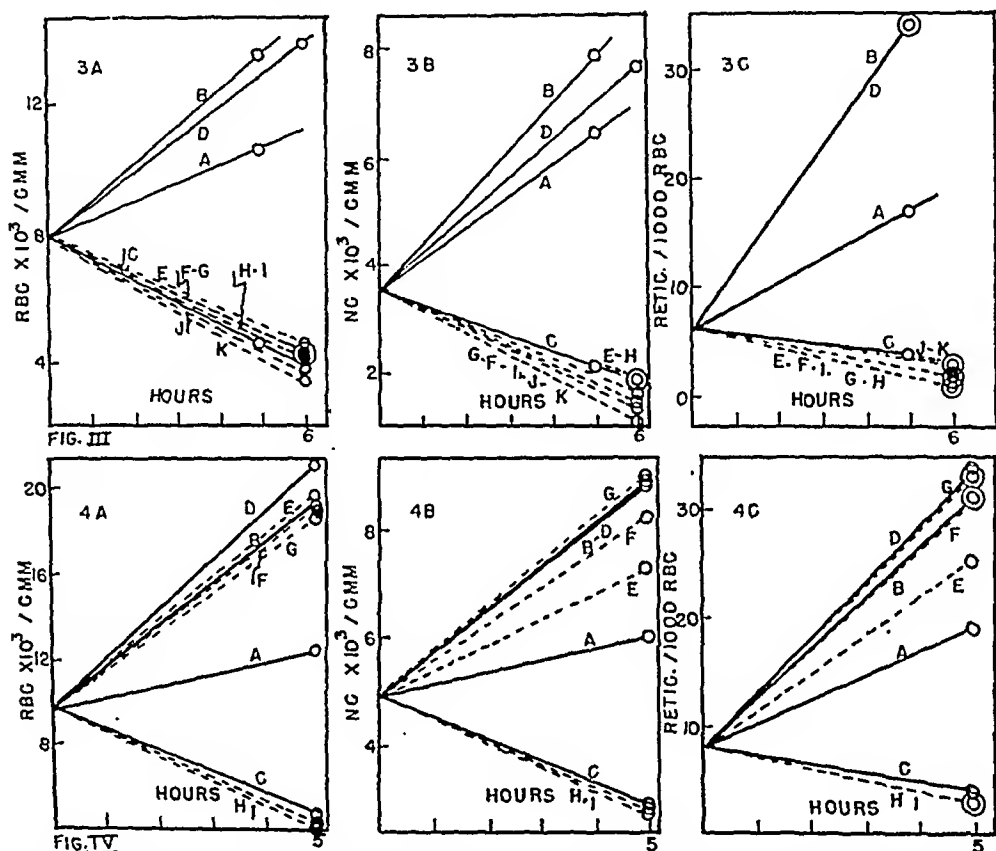


Fig. 3. EFFECT OF PTERIDINES and blood serum upon the rate of cell proliferation in bone marrow culture *in vitro*. The curves represent the response with the following supplements, including 0.1 ml. of blood serum from the conditions given: A, no supplement; B, 5 γ of xanthopterin per ml.; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine; D, normal blood serum; E, carcinoma of the breast; F, adenocarcinoma of the rectum; G, chondrosarcoma; H, cordoma of the sacrum; I, squamous cell carcinoma; J, pernicious anemia; K, pernicious anemia.

Fig. 4. EFFECT OF PTERIDINES and blood serum upon the rate of cell proliferation in bone marrow culture *in vitro*. The following supplements were added, including 0.1 ml. of blood serum from the conditions given: A, no supplement; B, 5 γ of xanthopterin per ml.; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; D, normal blood serum; E, F and G, active tuberculosis; H and I, broncheogenic carcinoma.

tion approximately equal to that produced by 5 γ of xanthopterin per ml. of bone marrow suspension. After inoculation with Brown-Pearce tumor there was a decrease in response which changed to an inhibition of cell proliferation. Ten days after inoculation the rabbit serum gave a response approximately equivalent to 5 γ of an antixanthopterin.

Cancer blood sera apparently contain an excess of a substance which will in-

hibit cell proliferation in bone marrow and normal tissue cell culture. As the effect may be counteracted or reversed by xanthopterin, the effect has been referred to as

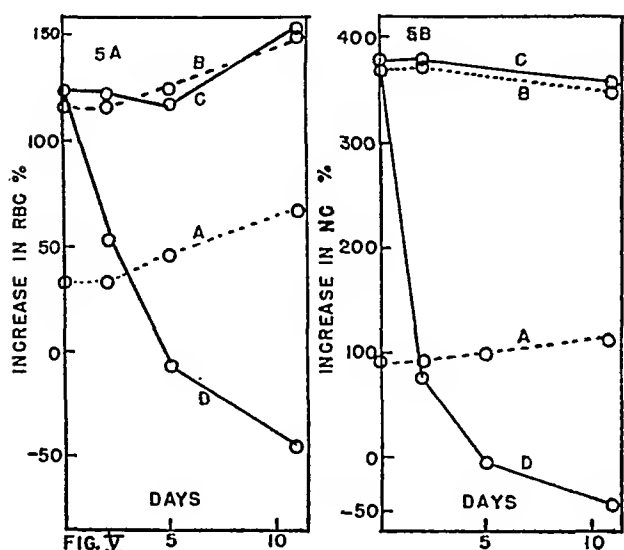


Fig. 5. EFFECT OF NEOPLASTIC DISEASE upon the response obtained when blood serum is added to bone marrow cultures. The following supplements were added, including 0.1 ml. of rabbit blood serum as given. The results are indicated as the per cent increase during a five-hour incubation: A, no supplement; B, 5 γ of xanthopterin per ml.; C, normal rabbit serum; D, blood serum of rabbits inoculated on zero day with Brown-Pearce Tumor.

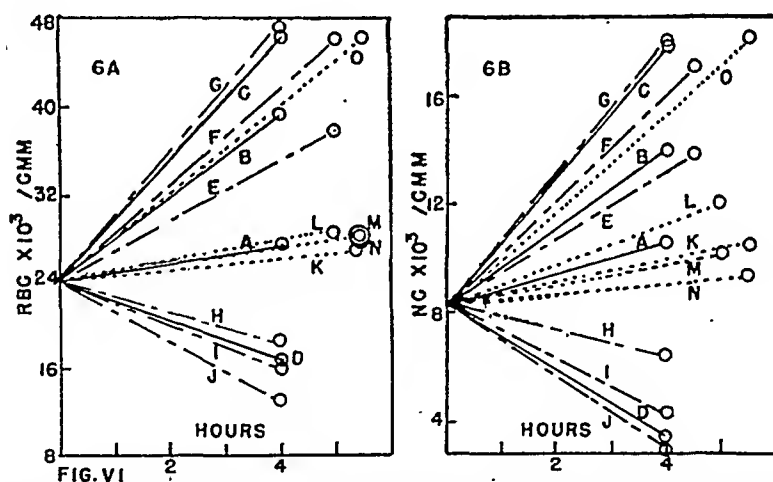


Fig. 6. EFFECT OF PTERIDINES and rabbit blood serum on the rate of cell proliferation in bone marrow cultures. The following supplements were added, including rabbit blood serum and serum from rabbits with Brown-Pearce tumor (B. P.) serum: A, no supplement; B, 2 γ of xanthopterin per ml.; C, 5 γ of xanthopterin per ml.; D, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; E, 0.05 ml. of normal rabbit serum; F, 0.1 ml. of normal rabbit serum; G, 0.2 ml. of normal rabbit serum; H, 0.05 ml. of B. P. rabbit serum; I, 0.1 ml. of B. P. rabbit serum; J, 0.2 ml. of B. P. rabbit serum; K, 0.1 ml. of normal rabbit serum and 0.1 ml. of B. P. rabbit serum; L, 0.2 ml. of normal rabbit serum and 0.1 ml. of B. P. rabbit serum; M, 0.1 ml. of B. P. rabbit serum and 5 γ of xanthopterin per ml.; N, 0.2 ml. of B. P. rabbit serum and 10 γ of xanthopterin per ml.; O, 0.2 ml. of normal rabbit serum and 0.1 ml. of B. P. rabbit serum.

that of an antixanthopterin. Figure 6 shows that the response to both normal (curves E, F, and G) and neoplastic (curves H, I, and J) serum is proportional to the amount of serum. The response with xanthopterin and an antixanthopterin is

given for comparison. Curves K, L and O of figure 6 show that the stimulating substance of normal rabbit serum and the inhibiting substance of the serum of tumor-bearing rabbits counteract each other in the same manner as do xanthopterin and antixanthopterin.

One tenth ml. of tumor rabbit serum is counteracted by 5 γ of xanthopterin, curve M, and 0.2 ml. is counteracted by 10 γ of xanthopterin per ml. of suspension, curve N.

All normal blood sera and blood sera from individuals or experimental animals with neoplastic disease do not exactly counteract each other in equal concentrations as in the example above. Both groups of sera give a range of response in bone marrow culture. Human normal blood sera and sera from cases of neoplastic disease counteract each other proportionally to the individual effect of each on the rate of cell proliferation.

There is present in normal blood serum a predominance of a substance which accelerates the rate of cell proliferation of normal bone marrow or tissue cells *in vitro*. There is present in the serum from individuals with neoplastic disease a predominance of a substance which inhibits cell proliferation in normal bone marrow and tissue cell cultures *in vitro*. It is probable that both a stimulating and an inhibiting substance is present in all sera and the effect observed is due to the relative concentrations at any given time or in an individual serum.

SUMMARY

Normal blood serum accelerates the rate of cell proliferation in bone marrow cultures *in vitro*. The blood serum from cases of pernicious anemia, leukemia and neoplastic disease inhibits cell proliferation in bone marrow cultures *in vitro*. The accelerating substance present in normal serum and the inhibiting substance present in blood serum from neoplastic disease counteract each other in their effect on cell culture *in vitro*.

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EFFECT OF XANTHOPTERIN AND OTHER PTERIDINES ON THE RATE OF CELL PROLIFERATION IN TISSUE CELL CULTURES

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IN 1939 Koscharka and Haug (1) reported that xanthopterin (uropterin) was widely distributed in animal tissues and suggested that it had some function in metabolism which they believed closely related to the function of riboflavin. Xanthopterin stimulates hemapoiesis and Norris and Majnarich (2) have shown that xanthopterin within a narrow range of concentration will increase the rate of proliferation of both red blood cells (RBC) and nucleated cells (NC) in bone marrow cultures *in vitro*. Other pteridines have been studied (3), but none found which has a greater stimulating effect than xanthopterin. Two of the pteridines studied, 2-amino-4-hydroxy-7-methyl pteridine and xanthopterin-7-carbonic acid, were found to have a strong inhibitory action on the cell proliferation in bone marrow cultures.

The present investigation is a study of the effect of xanthopterin and other pteridines on cell proliferation in a suspension of cells from tissues other than bone marrow.

To prepare a uniform suspension of cells, portions of fresh tissue were disintegrated in a small stainless steel cup for the Waring blender as described by Hoffstadt and Tripi (4). The tissue was obtained soon after death and cleaned of fat and extraneous material. The cleaned tissue was placed in the sterilized stainless steel cup together with Tyrode's solution without glucose. After running the blender for about one minute the resulting suspension was filtered through sterile gauze giving a uniform suspension of single cells. The suspensions of cells in Tyrode's solution without glucose were treated in much the same manner as the bone marrow suspensions previously described (2, 3). Two ml. of the suspension were placed in sterile 5 ml. vials containing a glass bead. Amounts of solutions containing the nutrients and supplements to be studied were added, such that the total volume of material in each vial of a given series was uniform. The vials were sealed with a sterile rubber cap and rocked slowly in a water bath at 37°C. Aliquots were withdrawn at intervals by means of a sterile needle and cell counts made using the usual technique for counting white blood cells or the nucleated cells of bone marrow cultures. The results obtained in a few representative experiments are given in figure 1. While the suspensions contained single cells there may be more than one type of cell in a suspension. The glandular tissues especially may contain different types of cells. Each vial contained 10 mgm. per ml. of acid-hydrolyzed casein, added as neutralized commercial casein hydrolysate, 0.5 mgm. per ml. of tryptophane and supplements as indicated.

All vials of a single experiment were prepared from aliquots of a uniform suspension of cells and the initial counts checked within the limits of error of counting technique. The conditions of the experiment were maintained uniform and after incubation the vials on each supplement checked within the limits of error in counting. In a representative experiment on cells of beef thyroid gland six counts on the

initial suspension gave an average of 4073 ± 150 cells per cmm. After incubation the vials without supplement had an average value of 4960 cells per cmm. With 5 γ per ml. of xanthopterin an average value of 7800 cells per cmm. with a standard error of 173 and a calculated significance factor t^1 compared to no supplement of 11.7 was obtained. Five gamma per ml. of 2-amino-4-hydroxy-7-methyl pteridine gave an average of 3400 cells per cmm. with a standard error of 156 and a t value of 6.7. Five gamma of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine gave an average of 4760 with a standard error of 89 and a t value compared to no

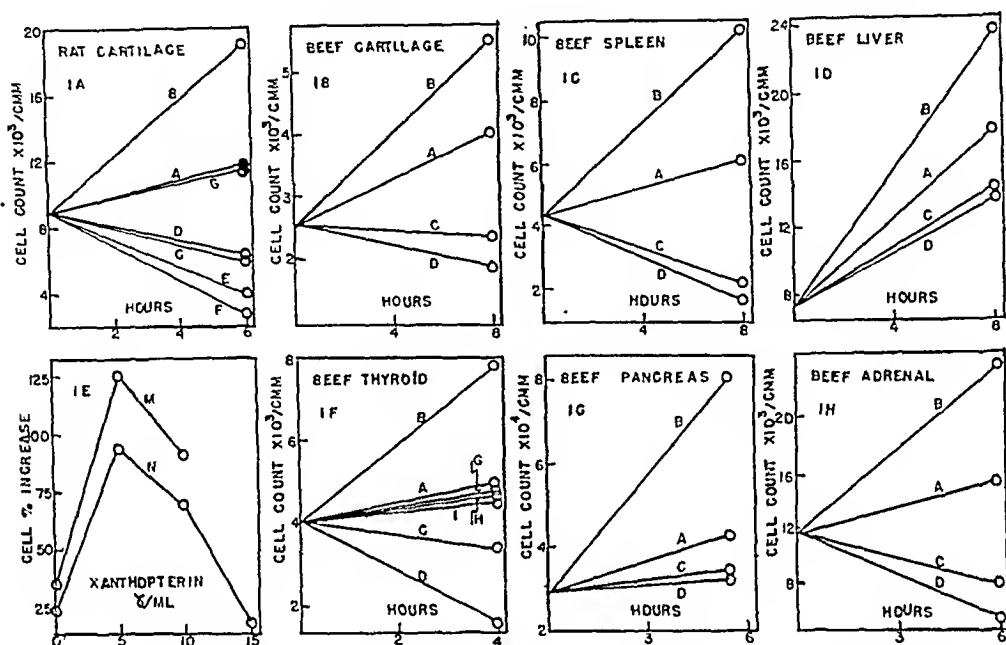


Fig. 1. EFFECT OF PTERIDINES upon the rate of cell proliferation in *in vitro* cultures of tissue cells. In graphs of the various tissues as indicated: Curve A has no supplement; curve B, 5 γ per ml. of xanthopterin; curve C, 5 γ per ml. of 2-amino-4-hydroxy-7-methylpteridine; curve D, 5 γ per ml. of xanthopterin-7-carboxylic acid; curve E, 10 γ per ml. of 2-amino-4-hydroxy-7-methylpteridine; curve F, 10 γ per ml. of xanthopterin-7-carboxylic acid; curve G, 5 γ per ml. of xanthopterin and 5 γ per ml. of 2-amino-4-hydroxy-7-methylpteridine; curve H, 10 γ per ml. of xanthopterin and 10 γ per ml. of 2-amino-4-hydroxy-7-methylpteridine; curve I, 5 γ per ml. of xanthopterin and 5 γ per ml. of xanthopterin-7-carboxylic acid.

Graph I-E, the influence of varying concentration of xanthopterin on the rate of cell proliferation; curve M, rat cartilage cells; and curve N, beef thyroid cells.

supplement of 1.02, indicating that the two pteridine derivatives had counteracted each other and given essentially the same value as with no supplement.

Under the conditions of the experiment, cell proliferation was observed in all

¹ The following formulae were used for calculation: standard deviation $\sigma = \sqrt{\frac{\sum(\bar{x} - x)^2}{n - 1}}$, where x is an individual count and \bar{x} is the average of n determinations in a given series. The standard error of the mean $\epsilon = \frac{\sigma}{\sqrt{n}}$. The significance factor, $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$, in which \bar{x}_1 is the average result of one group and ϵ_1 is its standard error, \bar{x}_2 is the average result from the other group and ϵ_2 is its standard error.

of the control groups of vials without added supplement. The rate of growth in the control groups varied with the type of tissue cells and was greatest for liver cells. The rate of cell proliferation was accelerated in each tissue cell suspension by xanthopterin and inhibited by 2-amino-4-hydroxy-7-methyl pteridine and xanthopterin-7-carboxylic acid. As the inhibitory effect of the last two pteridines is counteracted by xanthopterin, the effect may be referred to here as an antixanthopterin effect.

The optimum concentration of xanthopterin, for the cell proliferation *in vitro* in normal tissue cell suspensions studied, was 5 γ per ml., as is shown in figure 1E for beef thyroid gland cell suspension and rat cartilage cell suspension, and in figure 3 for a rat connective tissue cell suspension.

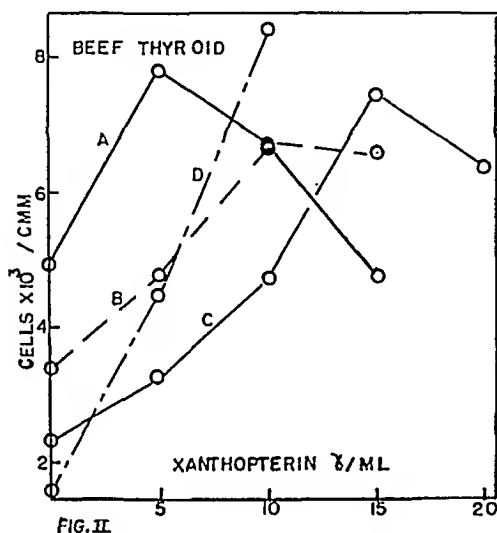


FIG. II

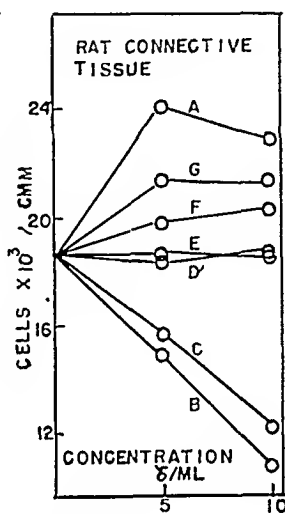


FIG. III

Fig. 2. ANTAGONISTIC EFFECT of xanthopterin and antixanthopterins on the cell proliferation of beef thyroid cells *in vitro*. Curve A is without 2-amino-4-hydroxy-7-methylpteridine (7-MP); curve B is with 5 γ per ml. of 7-MP; curve C is with 10 γ per ml. of 7-MP; curve D is with 5 γ per ml. of xanthopterin-7-carboxylic acid.

Fig. 3. EFFECT OF PTERIDINE and pteroyl derivatives upon the rate of cell proliferation *in vitro* of rat connective tissue cells. The supplements used were: curve A, xanthopterin; curve B, 2-amino-4-hydroxy-7-methylpteridine; curve C, xanthopterin-7-carboxylic acid; curve D, pteroylglutamic acid (folic acid); curve E, pteroyldiglutamyl glutamic acid (teropterin); curve F, pterioic acid; curve G, leucopterin.

A mutual antagonism between xanthopterin and 2-amino-4-hydroxy-7-methyl pteridine has been previously shown for bone marrow cultures (3), such that if both substances are present in the same concentration they counteract the effect of each other. Curve G in figure 1A and curves G, H and I in figure 1F illustrate the same type of antagonism for tissue cell cultures. Concentrations of 5 γ and 10 γ of both substances give the same rate of cell proliferation as with no supplement.

The effect of the balance in concentration of xanthopterin and antixanthopterins is also shown in figure 2. The apparent or observed optimum concentration of xanthopterin for cell proliferation is shifted by the addition of an antixanthopterin. Ten gamma per ml. of 2-amino-4-hydroxy-7-methyl pteridine is strongly inhibitory, and 15 γ per ml. of xanthopterin is also inhibitory, but together they give a stimulation of cell proliferation of thyroid cells equivalent to 5 γ per ml. of xanthopterin.

In addition to xanthopterin and an inhibitory pteridine, figure 3 shows that folic acid (pteroylglutamic acid) and teropterin (pteroyltriglutamic acid) have no effect upon cell proliferation of tissue cells *in vitro*. Pteric acid and leucopterin gave a slight stimulation of cell proliferation, but much less than xanthopterin. The effect of leucopterin and pteric acid may be due to partial conversion to xanthopterin.

SUMMARY

Tissue cells were cultured *in vitro* for study. Xanthopterin increased the rate of cell proliferation in all tissue cell cultures studied. Two-amino-4-hydroxy-7-methyl pteridine and xanthopterin-7-carboxylic acid had an inhibiting effect on cell proliferation in tissue cell cultures which was counteracted by xanthopterin.

There was a mutual counteracting effect in tissue cell cultures of xanthopterin and the inhibiting pteridines, which canceled the effect of each other on cell proliferation when both were present in the same concentration. When present in unequal concentration the effect of the substance present in excess was observed. The intensity of the effect observed was proportional to the excess of one substance beyond that balanced in the solution by an equal amount of the other.

Pteroylglutamic acid (folic acid) and pteroyltriglutamic acid (teropterin) had no effect upon the rate of tissue cell proliferation *in vitro*.

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EFFECT OF PTERIDINES AND BLOOD SERUM ON NEOPLASTIC CELL CULTURE *IN VITRO*

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CERTAIN pteridines and blood sera have been found to have a very marked effect upon the rate of cell proliferation in bone marrow and tissue cell cultures *in vitro*, (1, 2). Of the pteridines tested upon normal bone marrow and tissue cell preparations, xanthopterin had the greatest stimulating effect and 2-amino-4-hydroxy-7-methyl pteridine and xanthopterin-7-carboxylic acid strongly inhibited cell proliferation. Normal blood serum has a stimulating effect similar to xanthopterin on bone marrow and tissue cell cultures *in vitro*. Blood serum from neoplastic disease, leukemia and pernicious anemia have an inhibiting effect (2) upon cell proliferation *in vitro* similar to the effect of an antixanthopterin.

In the present paper the effect of pteridines and blood serums on the rate of proliferation *in vitro* of the cells of neoplastic growth is reported.

Cell suspensions of the neoplastic tissue were prepared in a manner similar to the preparation of cell suspensions of normal tissue (2). As soon as possible after removal of the tissue, it was disintegrated in a sterilized stainless steel cup (3) for the Waring blender. Tyrode's solution without glucose was added to the tissue. After disintegration the suspension was filtered through sterile gauze and the suspension of cells used in cell culture experiments. The technique used in culturing the cell suspension was the same as that previously described for bone marrow cultures and normal tissue cell cultures (1, 2). Ten mgm. of casein hydrolysate and 0.5 mgm. of tryptophane were added per ml. of cell suspension. Supplements were added as indicated in the experiments, and the cell suspensions incubated at 37°C.

Figure 1 shows the response obtained with rabbit Brown-Pearce tumor cells. The effect of pteridines is opposite to that obtained with normal cells as shown in figure 1A. The cell proliferation of the cancer cells is strongly inhibited by xanthopterin as shown by curves B, C and D. The rate of cell proliferation is increased by an antixanthopterin as shown in curve E for 2-amino-4-hydroxy-7-methyl pteridine. Xanthopterin and antixanthopterin counteract the effect of each other in the response with neoplastic tissue cells as with normal cells, curves F, G, H and I.

Figure 1B shows the counteracting effect of xanthopterin and antixanthopterin, which is very similar to the type of response obtained with normal cells (1, 2) except that the pteridines have the opposite effect. The apparent stoichiometric relationship in antagonism is present as it is with normal cells.

Figure 1C shows the effect of normal sera and sera from neoplastic disease compared with that of the pteridines. Folic acid has no effect as shown in curve I. One-tenth ml. of normal serum inhibits cell proliferation of cancer tissue cells *in*

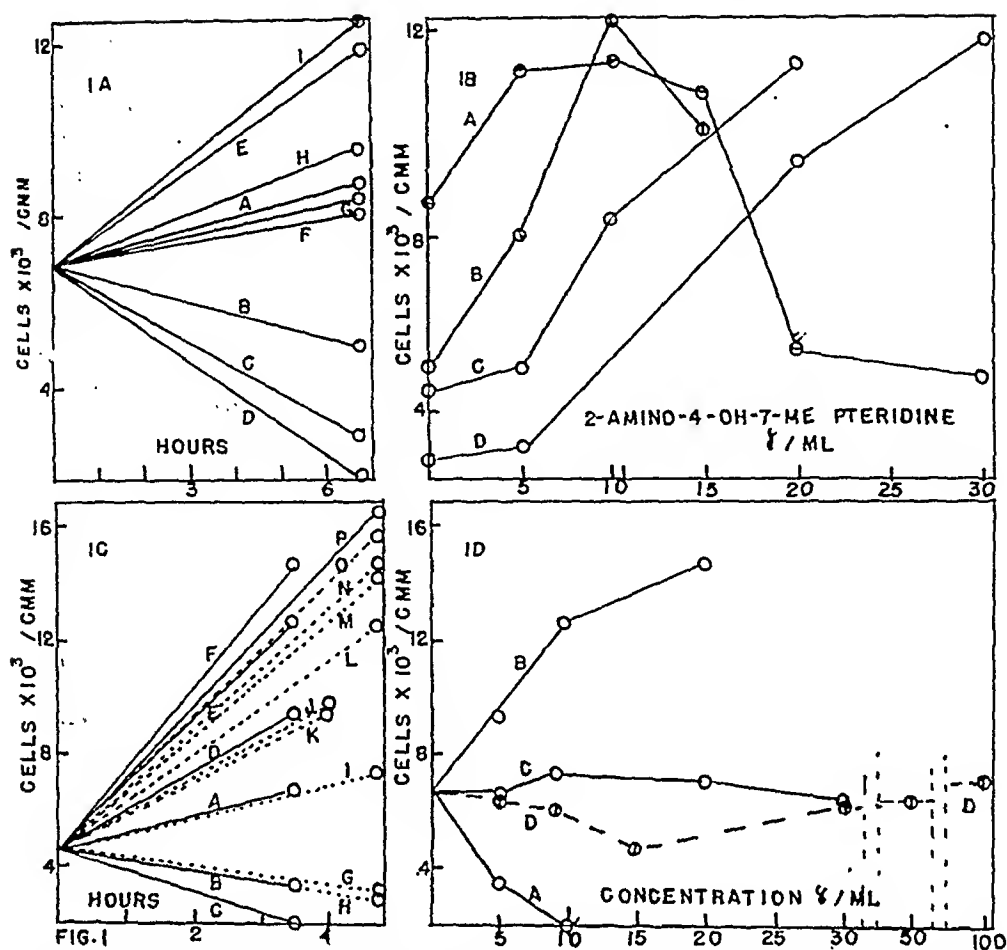


Fig. 1. THE EFFECT OF PTERIDINES and blood serum on the rate of cell proliferation of Brown Pearce tumor cells *in vitro*. Fig. 1A. The following supplements were added: A, no supplement; B 5 γ of xanthopterin per ml.; C, 20 γ of xanthopterin per ml.; D 30 γ of xanthopterin per ml.; E, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; F, 5 γ of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; G, 10 γ of xanthopterin and 10 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; H, 20 γ of xanthopterin and 20 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; I, 20 γ of xanthopterin and 30 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.

Fig. 1B. The balance in counteracting effect of xanthopterin and antixanthopterin on the rate of cell proliferation of Brown-Pearce tumor cells *in vitro*. Each curve represents the following concentration of added xanthopterin per ml. of cell suspension: A, none; B, 5 γ ; C, 10 γ ; D, 20 γ .

Fig. 1C. The following supplements were added including 0.1 ml. of blood serum from the conditions given: A, no supplement; B, 5 γ of xanthopterin per ml.; C, 10 γ of xanthopterin per ml.; D, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; E, 10 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; F, 20 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; G, and H, normal human blood serum; I, pteroyl glutamic acid (folic acid); J and K, pernicious anemia; L, carcinoma of the rectum; M, chondrosarcoma; N, squamous cell carcinoma; O, carcinoma of the breast; P, adenocarcinoma of the rectum.

Fig. 1D. Effect of varying concentration of the following supplements: A, xanthopterin; B, 2-amino-4-hydroxy-7-methyl pteridine; C, folic acid (pteroyl glutamic acid); D, teropterin.

in vitro, to an extent about equivalent to that of 5 γ of xanthopterin per ml. of suspension as shown by curves G and H.

The blood serum from persons with pernicious anemia and neoplastic disease increases the rate of proliferation of cancer cells *in vitro*, as shown by curves J to P, which may be compared with curves D, E and F.

Figure 1D shows that folic acid and teropterin have little or no effect upon the rate of cell proliferation in cancer cell cultures *in vitro*.

A spontaneous neoplastic growth appeared on one of the colony rats. The tissue of the rat neoplasm was cultured by the technique described above and the results are given in figure 2. The responses to pteridines, normal serum and serum

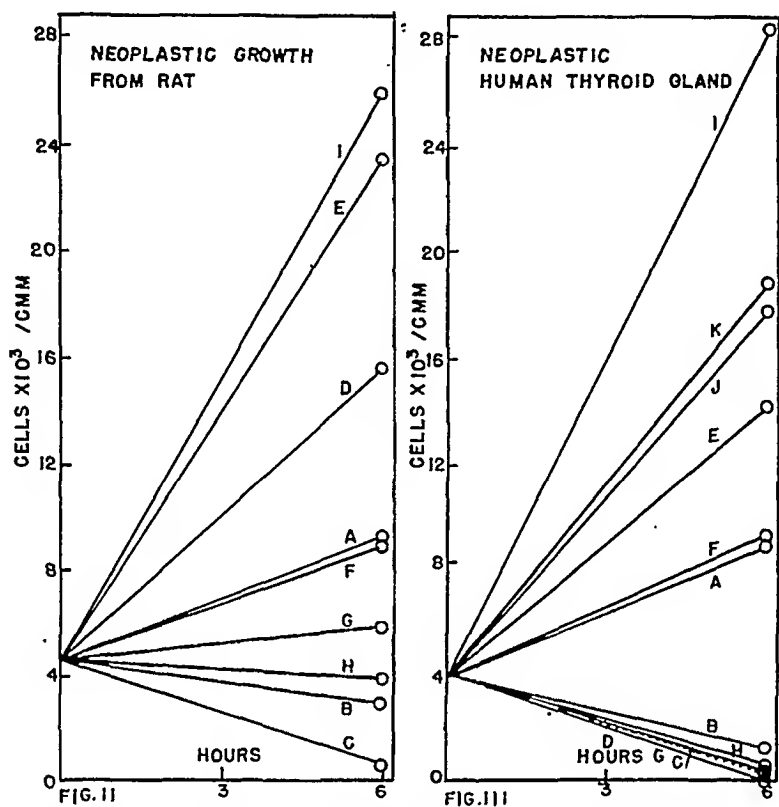


Fig. 2. EFFECT OF PTERIDINES and blood serums on the rate of cell proliferation *in vitro* of the cells of a neoplastic growth from a rat. The following supplements were added, including 0.1 ml. of blood serum as given: A, no supplement; B, 5 γ of xanthopterin per ml.; C, 10 γ of xanthopterin per ml.; D, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; E, 10 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; F, 5 γ of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; G, 10 γ of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; H, normal human blood serum; I, human cancer serum.

Fig. 3. EFFECT OF PTERIDINES and blood serums on the rate of cell proliferation *in vitro* of the cells of a neoplastic growth from a human thyroid gland. The following supplements were added, including 0.1 ml. of the blood serum given: A, no supplement; B, 1 γ of xanthopterin per ml.; C, 5 γ of xanthopterin per ml.; D, 15 γ of xanthopterin per ml.; E, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; F, 5 γ of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; G, 10 γ of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine per ml.; H, normal human serum; I, human cancer serum; J, pernicious anemia; K, myeloid leukemia.

from neoplastic disease are the same as for the Brown-Pearce tumor cells reported above and opposite to those observed with normal tissue.

Figure 3 shows the response with the cultured cells of a neoplastic growth of a human thyroid gland removed by surgery. Cell proliferation *in vitro* is retarded by xanthopterin and by normal blood serum.

The rate of cell proliferation in suspensions of neoplastic tissue cells is accelerated by an antixanthopterin and by the blood serum from neoplastic disease, leukemia and pernicious anemia. Xanthopterin and 2-amino-4-hydroxy-7-methyl pteridine counteract the effect of each other in equal concentrations.

There are distinctly two types of cells; first, those in which the rate of cell proliferation *in vitro* is accelerated by xanthopterin and inhibited by antixanthopterins, represented by normal bone marrow and tissues, and second, those cells in which cell proliferation is inhibited by xanthopterin and increased by antixanthopterins, represented by the cells of neoplastic growth. The cells of bakers yeast belong to the first type.

SUMMARY

Xanthopterin strongly inhibits the cell proliferation *in vitro* of cells of neoplastic growth. Antixanthopterins accelerate the rate of cell proliferation *in vitro* of cells of neoplastic growth. Xanthopterin and antixanthopterin counteract the effect of each other in equal concentration on the cells of neoplastic growth *in vitro*.

Normal blood serum has an inhibiting effect upon the cell proliferation of the cells of neoplastic growth *in vitro*. The blood serum from individuals or experimental animals with neoplastic disease increases the rate of cell proliferation in cultures of cells of neoplastic growth *in vitro*.

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EFFECT OF PTERIDINES AND BLOOD SERA ON HUMAN BONE MARROW CELLS *IN VITRO*

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PTERIDINES and blood sera have been shown to have a marked effect upon cell proliferation *in vitro* of bone marrow cell suspensions of the rat, rabbit, cat, sheep and beef (1, 2). A study has been made of the effect of pteridines

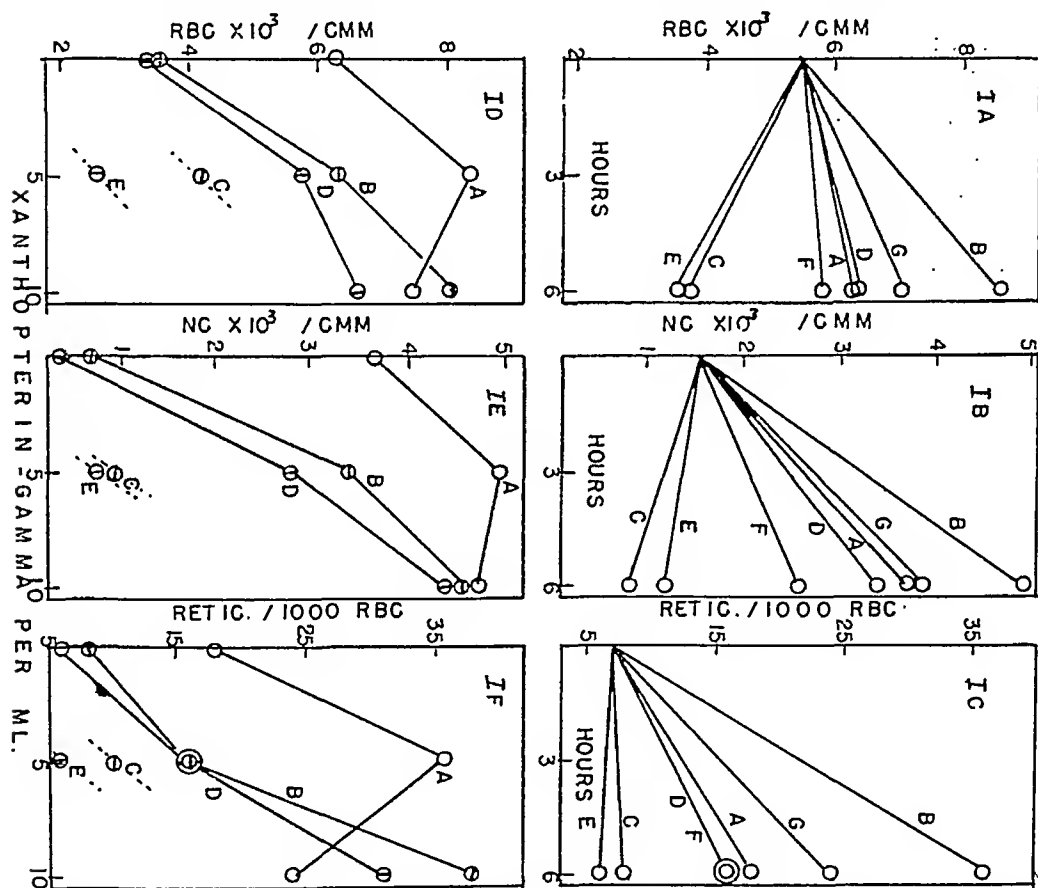


Fig. 1, parts A, B and C. EFFECT OF PTERIDINES on the rate of cell proliferation *in vitro* in human bone marrow suspension. The following supplements were added to the suspension: A, nothing; B, 5 γ of xanthopterin/ml.; C, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine/ml.; D, 5 γ of xanthopterin and 5 γ of 2-amino-4-hydroxy-7-methyl pteridine/ml.; E, 5 γ of xanthopterin-7-carboxylic acid/ml.; F, 5 γ of xanthopterin and 5 γ of xanthopterin-7-carboxylic acid/ml.; G, 10 γ of pterioic acid/ml.

Parts D, E, and F. The counteracting effect of xanthopterin and antixanthopterin on the rate of cell proliferation *in vitro* in human bone marrow suspension. The curves represent the following supplements: A, no antixanthopterin; B, 5 γ of 2-amino-4-hydroxy-7-methyl pteridine/ml.; C, 10 γ of 2-amino-4-hydroxy-7-methyl pteridine/ml.; D, 5 γ of xanthopterin-7-carboxylic acid/ml.; E, 10 γ of xanthopterin-7-carboxylic acid/ml.

Received for publication June 1, 1948.

and blood sera on the rate of cell proliferation *in vitro* of a suspension of human bone marrow cells.

A portion of human rib bone was obtained from surgery as it was removed in an operation for lobectomy. A suspension of the bone marrow cells was prepared in Tyrode's solution without glucose. The cell suspension was cultured by the technique previously described (1, 2). Ten mgm. of casein hydrolysate and 0.5 mgm. of tryptophane were added per ml. of cell suspension. Supplements were added as in-

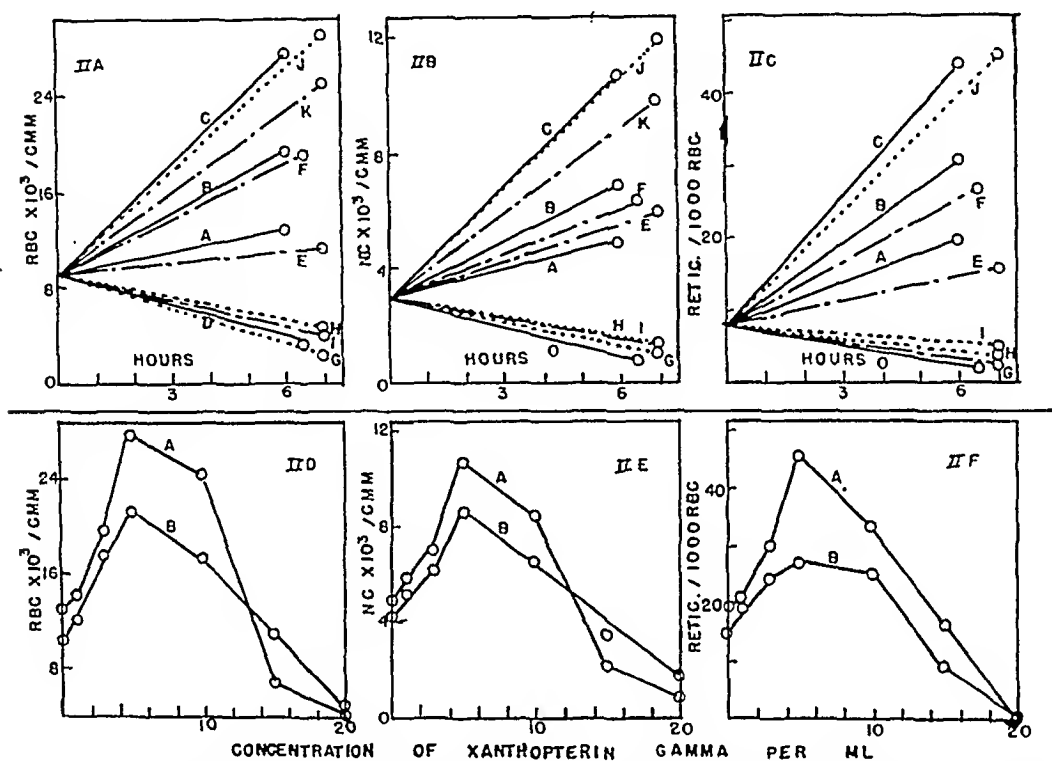


Fig. 2, parts A, B and C. EFFECT OF PTERIDINES and blood sera on the rate of cell proliferation *in vitro* in human bone marrow suspension. The following supplements were added: A, none; B, 3 γ of xanthopterin/ml.; C, 5 γ of xanthopterin/ml.; D, 5 γ of 2-amino-4-hydroxy-6-methyl pteridine/ml.; E, 10 γ of folic acid/ml.; F, 10 γ of pteric acid/ml.; G, 5 γ of xanthopterin-7-carboxylic acid/ml.; H, 0.1 ml. of human blood serum from a case of cancer; I, 0.1 ml. of human blood serum from a case of myelogenous leukemia; J, 0.1 ml. of normal human blood serum; K, 5 γ of leucopterin/ml.

Parts D, E and F. Effect of varying concentration of xanthopterin on the rate of cell proliferation *in vitro* in human bone marrow suspension. A, 6 hours incubation; B, 4 hours incubation.

indicated and the rate of cell proliferation observed by making cell counts after periods of incubation at 37°C. The results are shown in figures 1 and 2.

Figure 1, parts A, B and C, shows that xanthopterin increases the rate of cell proliferation *in vitro*, in a human bone marrow suspension of both red blood cells (RBC) and total nucleated cells (NC). Curves C and E show the inhibiting effect of the antixanthopterins, 2-amino-4-hydroxy-7-methyl pteridine and xanthopterin-7-carboxylic acid. The inhibiting effect of antixanthopterin is counteracted by xanthopterin when present at the same concentration as shown in curves D and F. The counteracting effect of xanthopterin and antixanthopterins is represented in figure 1, parts D, E, and F.

The optimum concentration of xanthopterin for cell proliferation *in vitro* of human bone marrow is approximately 5 γ per ml. of suspension. Folic acid (pteroyl-glutamic acid) has practically no effect upon cell proliferation. Leucopterin increases the rate of cell proliferation but less than xanthopterin.

Normal human blood serum, *curve J*, increases the rate of cell proliferation *in vitro* of human bone marrow cells approximately equivalent to 5 γ per ml. of xanthopterin. The blood serum of persons with cancer, *curve H*, or with myelogenous leukemia, *curve I*, inhibit cell proliferation in human bone marrow cultures.

The cells of human bone marrow respond to the pteridines and to blood sera in the same manner as the cells of bone marrow suspensions of experimental animals.

SUMMARY

Xanthopterin and normal blood serum increase the rate of cell proliferation *in vitro* in human bone marrow suspensions. Antixanthopterins and blood serum from individuals with cancer or leukemia inhibit cell proliferation *in vitro* in human bone marrow suspensions. Xanthopterin and antixanthopterin counteract the effect of each other on human bone marrow suspensions.

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ESTIMATION OF CHANGES IN PLASMA AND EXTRACELLULAR FLUID VOLUME FOLLOWING CHANGES IN BODY CONTENT OF WATER AND CERTAIN SOLUTES, BY MEANS OF AN OSMOMETRIC EQUATION¹

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INVESTIGATORS (1-6) have employed or confirmed, for particular experimental conditions, the hypothesis that the concentration of total base throughout the body water is approximately the same and that change in any portion is approximately equalled in all other portions. It has also been experimentally verified that changes in compartmental volumes are interrelated, e. g., under specific conditions of water loss or gain with or without loss or gain of osmotically active substances confined to the extracellular space. Lacking thus far is an integrated picture of the consequences of this hypothesis under all conditions in which the body might behave as a 'perfect osmometer.'

It is often of interest to compare the actual effect of some procedure designed to alter plasma volume or extracellular volume with a value expected on the basis of osmotic equilibrium. Differences between experimental and calculated values serve to stimulate further inquiry since no large body of evidence indicates the gross untenability of the osmometer hypothesis. On the basis of this hypothesis, expected increments to plasma volume or extracellular volume are readily obtained by the following osmometric equation (8) or its family of curves (fig. 1).

The derivation of this equation, or the osmometer hypothesis itself, is based on several assumptions. First, a load of water, whether positive or negative, is fractionally distributed among plasma, interstitial, and intracellular compartments in proportion to their actual volumes so that the ratio of increment to initial compartment volume is constant. Second, a load of solute, whether positive or negative, is confined to the extracellular space for the period of equilibration and is distributed between the plasma and interstitial volumes similarly in proportion to their actual volumes. Hetherington (7) has established the time for equilibration of a salt load in the cat as between 15 and 30 minutes, i. e., essential equilibrium obtains after this period. Third, the effective osmotic pressure within the intracellular space is equal to that osmotic pressure within the extracellular space at equilibrium. Fourth, the quantity of intracellular solute exerting effective osmotic pressure remains constant for the period of equilibration.

Where V_e = initial extracellular fluid volume in liters, V'_e = final extracellular

Received for publication March 29, 1948.

¹ This work was supported by a grant from the U. S. Public Health Service.

fluid volume in liters, V_i = initial intracellular fluid volume in liters, V'_i = final intracellular fluid volume in liters, W = initial total body water volume in liters,

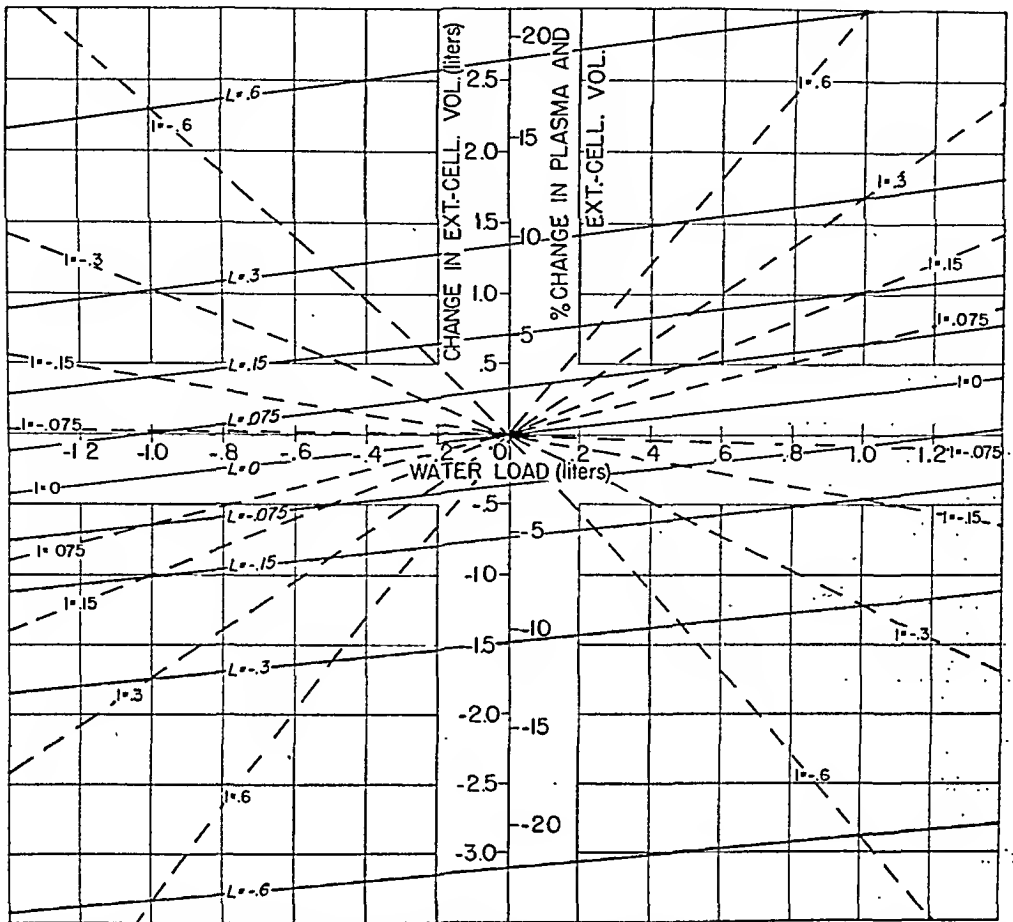


Fig. 1. RELATIONS BETWEEN CHANGE IN EXTRACELLULAR VOLUME (or % change in extracellular volume or of plasma volume) and load of water. Based on a 70 kgm. man containing 14 l. of extracellular fluid and 49 l. of total body water. The hatched lines passing through the origin represent concentrations in equivalents per l. ($I = L/L_{H_2O}$) of administered or withdrawn NaCl solutions, relating change in extracellular volume to volume of administered or withdrawn solution. At high I values, appreciable deviations from rectilinearity appear. Each of the parallel lines crossing the coordinates represents a given load of NaCl in equivalents (L). By inspection we see that a water load of 1 l. containing a load of NaCl of 0.15 equivalents leads to an increment in the extracellular volume of 1 l. [Erect a perpendicular from the abscissal water load of +1.0 liter until it meets line $L = 0.15$. From this point (also common to the line $I = 0.15$) a perpendicular can be dropped to the ordinate at +1.0 liters change in extracellular volume.]

In the first and third quadrants the listed concentrations are positive since both salt and water loads are positive or negative at the same time. The second and fourth quadrants contain 'negative' concentrations where the loads of salt and of water have different signs at the same time.

(Theoretical reasons may be given why the change in plasma volume should not change strictly in proportion to the interstitial. It is in cognance of this and to point up the heuristic value of deviations that such changes are indicated on the graph, which would occur if they were simple osmometric ones.)

W' = final total body water volume in liters, A = initial concentration in extracellular and in intracellular compartments in osmols per liter, A' = final concentra-

tion in extracellular and in intracellular compartments in osmols per liter, L = load of solute in extracellular compartment in osmols, and L_{H_2O} = load of water in liters, then

$$W' = W + L_{H_2O}, \quad (1)$$

$$V_i = W - V_e, \quad (2)$$

$$V'_i = W' - V'_e, \quad (3)$$

$$\text{From the second assumption, } A' = \frac{V_e A + L}{V'_e}. \quad (4)$$

$$\text{From the fourth assumption, } A' = \frac{V_i A}{V'_i}. \quad (5)$$

TABLE 1¹

DATA OF ELKINTON AND WINKLER (8) AND OF WINKLER, ELKINTON, HOPPER, AND HOFF (5)					FROM EQUATION (8)	% DIFFERENCE
Dog. no.	Body wt.	L_{H_2O}	L	E'	V'_e	$100(E' - V'_e)/V'_e$
	kgm.	l.	equiv.	l.	l.	
16	6.16	+0.14	+0.214	2.27	2.35	-3.4
17	6.70	-0.32	+0.071	1.81	1.84	-1.6
18	5.50	-0.28	+0.081	1.58	1.57	+0.6
21	7.95	-0.37	+0.116	2.28	2.28	0.0
21	7.95	-0.26	-0.031	1.93	1.75	+10.0
11E	12.22	-0.31	+0.230	3.65	3.80	-3.9
11E	12.22	-0.62	-0.179	2.36	1.95	+21.0
11D	12.22	-0.18	-0.009	3.06	2.96	+3.4
27	7.85	-0.02	+0.366	3.33	3.09	+8.1
28	7.95	-0.40	+0.300	2.73	2.77	-1.4
Averages.....				2.50	2.44	

¹ L is the average of the loads of Na and of Cl; E' is the average of the final extracellular volumes calculated both from Na and from Cl loads and concentrations.

$$\text{Combining (2) and (3) with (5), } A' = \frac{(W - V_e)A}{W' - V'_e}. \quad (6)$$

$$\text{Combining (4) and (6), } \frac{V_e A + L}{V'_e} = \frac{(W - V_e)A}{W' - V'_e}. \quad (7)$$

Solving for V'_e and combining with (1),

$$V'_e = \frac{W'(V_e A + L)}{WA + L} = \frac{(W + L_{H_2O})(V_e A + L)}{WA + L}. \quad (8)$$

To the extent that the osmometer hypothesis is valid, equation 8 may be used to estimate changes in plasma or extracellular volume when the other factors in the equation are either measured or taken at commonly accepted normal values. It neglects tissue pressures, ordinarily insignificant. A plot of a few of the family of

curves generated by this equation is shown in figure 1. Concentrations and loads of solutes are generally best expressed in terms of osmols but in the case of NaCl the approximately 0.3 osmols per liter concentration of extracellular fluid may be taken as 0.15 equivalents per liter.

Application of the Equation. Elkinton and Winkler (8) and Winkler, *et al.* (5) give data on dogs subjected to varying loads of NaCl and water which permit calculation of final extracellular fluid volumes by means of *equation 8*. Their own calculations are based on an assumed original extracellular volume equal to 25 per cent of the body weight, and the formula they use has the form of *equation 4*.

$$E' = \frac{EA + L}{A'}, \quad (9)$$

where E and E' respectively are the initial and final extracellular volumes, A and A' are the initial and final measured concentrations of either Na or Cl in extracellular fluid and L is the load of either Na or Cl.

For purposes of comparison between E' as determined experimentally by the above workers and V_e solved from *equation 8*, V_e is taken as 25 per cent of the body weight and A is taken at their figure of approximately 0.14 equivalents per liter. Body water, W, is taken at 70 per cent of body weight (table 1).

The agreement (between 2.5 per cent; average deviation 4.9 per cent) between the method based on determining concentrations in extracellular water and the osmometric one proposed is not presumed to argue either in favor of the applicability of *equation 8* or of the precision of the data reviewed. The magnitude of the discrepancies is taken to indicate the degree to which the fluid compartmental system fails to behave as a perfect osmometer. The figures presented suggest that these particular compartmental exchanges parallel closely those of a perfect osmometer.

SUMMARY

An equation for a 'perfect osmometer' is derived, defining relations among volumes of extracellular and intracellular fluid, loads of certain solutes substantially confined to the extracellular space and loads of water. A family of curves defined by this equation is indicated graphically. Osmometric changes in extracellular volume following loads of water and salt are shown by sample computations from data in the literature.

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CAPILLARY PERMEABILITY: RATE OF TRANSCAPILLARY EXCHANGE OF IRON ADDED TO PLASMA AS RADIOACTIVE FERRIC BETA₁-GLOBULINATE

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ONE of our purposes in this series of studies is to characterize the capillary wall in terms of its relative permeability to the constituent substances of the blood plasma. Using the tracer technique, we have previously determined the rate of exchange, in the guinea pig, of sodium and water of plasma with that of extravascular fluid (1, 2). The present experiments have as their objective an evaluation of the rate of escape of iron from the plasma of the guinea pig. As shown by E. J. Cohn (3), iron in the plasma is present as ferric beta₁-globulinate. That iron crosses the vascular wall promptly is evident from the experiments of Granick and Hahn (4) and from our own observations on fetal uptake of iron from the maternal plasma (5). The state in which iron crosses the vascular wall from the plasma to the extravascular fluid is unknown. There is no evidence to contradict the view that it escapes from the plasma as the beta₁-globulinate but it is possible, until demonstrated otherwise, that iron is freed from this molecule within the endothelial cells of the capillaries and moves into the extravascular fluid in some other form. Except for purposes of discussion, we shall consequently make no assumption about the form in which iron crosses the capillary wall.

METHODS

In our experiments with deuterium oxide and with sodium and chloride tagged with a radioactive isotope, the experimental procedure consisted of intravenous injection of the tracer substance and determination of its subsequent decrease in concentration in the plasma. The same procedure is not necessarily valid with iron since the iron of the plasma is present not as ferric ion but as ferric beta₁-globulinate (3). Whether intravenous injection of a ferric salt, therefore, is acceptable depends upon the rapidity and completeness with which ferric ions combine with beta₁-globulin. Because of this uncertainty, our first effort was directed toward obtaining a reliable source of ferric globulinate labeled with radioactive iron. This was achieved by a procedure suggested by the experiments of Hahn and his associates (6). Guinea pigs weighing from 500 to 1000 grams were bled 5 to 10 ml. every other day by cardiac

Received for publication May 28, 1948.

puncture until 25 to 45 per cent of the calculated blood volume (1) had been withdrawn. In order to reduce the iron stores of the body, the animals were maintained on a diet relatively low in iron and calories (small amounts of cabbage and carrots) supplemented with vitamins for from 6 to 14 days following the first bleeding. From 1 to 1.5 mgm. of radioiron (Fe^*) as ferrous chloride was then introduced into the stomach by tube. Two and a half to three hours later the animal was bled with needle and syringe from a carotid artery and heparin added to the blood. From 6 to 10 ml. of plasma with about 2 micrograms of radioiron per ml. were obtained from each animal in this way.

Because the preparation of plasma containing radioiron in the manner described above is laborious and inconvenient, we attempted to find a simpler and equally acceptable method. Our criteria for an acceptable method were as follows: 1) the curve describing the disappearance of the tagged iron from the plasma of the transfused animal must have the characteristics of that obtained with the tagged ferric beta₁-globulinate synthesized by the anemic animal and 2) extrapolation of the disappearance curve to zero time must yield an initial, theoretical concentration of radioiron compatible with the value calculated from the total amount injected and the predicted plasma volume of the animal. In the method first attempted, radioiron as ferric chloride was added *in vitro* with vigorous stirring (in amounts varying from 1 to 10 micrograms per ml. of plasma) to plasma obtained by bleeding a guinea pig. This plasma was then injected into a carotid artery of a recipient and the loss of the radioiron from the plasma followed as a function of time. A typical experimental result with this method is shown in figure 1. There was a rapid loss of radioiron from the plasma during the first 20 or 30 minutes of the experiment. This was followed by a slow decrease in concentration; this second portion of the curve had the same characteristics as that observed with the tagged plasma synthesized by the anemic animal (compare fig. 2). The plasma volume obtained by extrapolation of the second portion of the curve to zero time was of course impossibly high; in the experiment of fig. 1, it amounted to 80 ml. whereas the anticipated volume on the basis of 4.3 ml. plasma per 100 gram body weight (1) was 34 ml. We suppose that when Fe^*Cl_3 is added to plasma *in vitro* only a part of the iron combines with globulin and the remainder, at the pH of the plasma, exists in some other form perhaps as the hydroxide and ascorbate. When this mixture of ferric compounds is injected into an animal, all but the globulinate are rapidly lost from the circulation, and after a short period the radioiron remaining in the plasma is present as the globulinate.

If this explanation is correct, it should be possible to prepare the globulinate by injecting a small amount of radioiron intravascularly into a donor and after an appropriate interval bleeding the animal for its plasma. This is the procedure which we have adopted. We have injected into the donor a maximum of 140 micrograms of Fe^* as ferric chloride, waited a minimum of 30 minutes, then bled the animal from the carotid artery. Plasma prepared in this way contains about 1 microgram of Fe^* per ml. and behaves on transfusion, into a recipient, just as does labeled plasma synthesized by the anemic animal. The rate of disappearance from the blood stream in both instances is alike and both preparations yield plasma volumes which are in the range calculated from data obtained with the dye T-1824 (1).

The plasma containing labeled ferric beta₁-globulinate was transfused into a recipient under nembutal and ether anesthesia. A common carotid artery of the recipient was exposed, the artery ligated high in the neck and a bull-dog clamp placed on a proximal portion. The wall of the carotid between the ligature and clamp was then incised but not severed and a No. 19 three-inch needle with blunt point introduced into the lumen of the artery. The needle was secured in place by a bull-dog

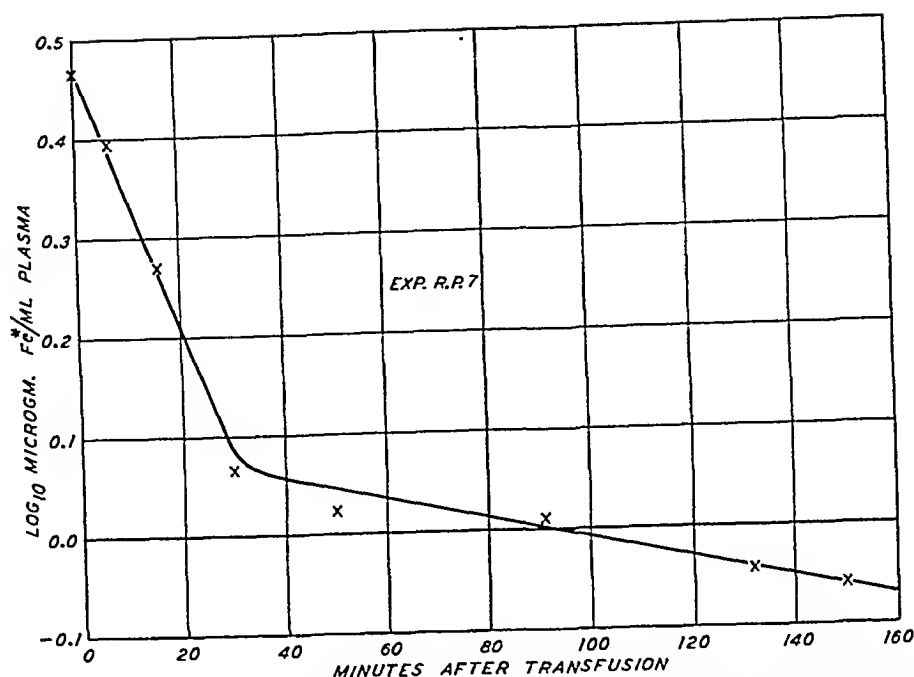


Fig. 1. LOSS OF RADIOIRON from plasma of recipient transfused with plasma to which radioiron was added *in vitro*. The value at zero time was calculated from the amount of Fe^* transfused and the plasma volume of the animal.

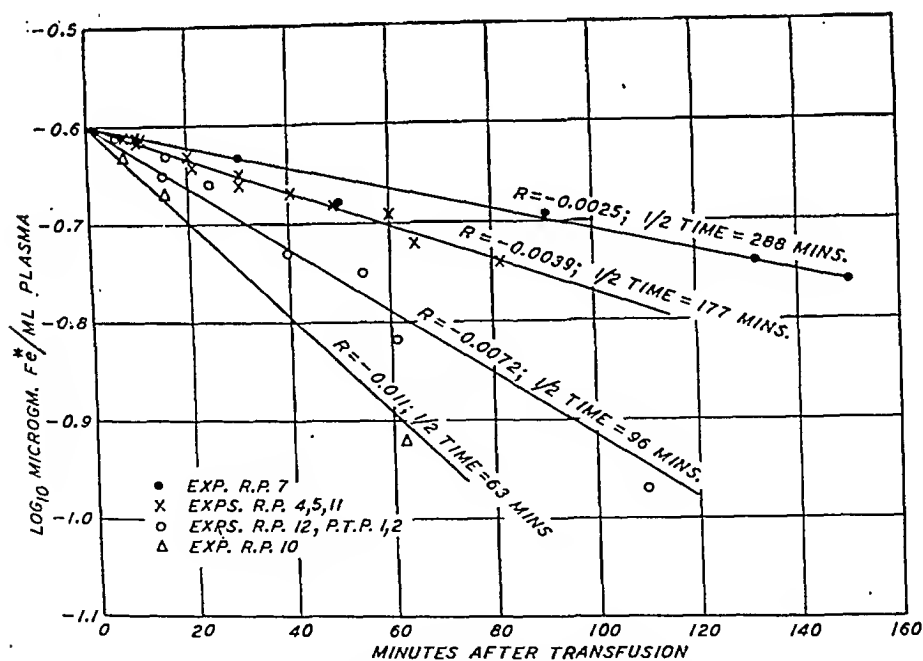


Fig. 2. CHANGE IN CONCENTRATION of labeled ferric β_1 -globulinate in the plasma with respect to time after intravascular injection. R has been derived by dividing the decrement for any period in the logarithm to the base 10 of the concentration of Fe^* by the corresponding time in minutes and multiplying by 2.3 to convert the logarithm to base e . Half-time is equal to the time necessary for half the Fe^* present at any instant to disappear from the plasma. Only the second rate of experiment RP7 has been plotted (cf. fig. 1).

clamp placed on the wall of the vessel around the needle. Five to 7 ml. of blood were withdrawn from the artery and the animal then transfused with 6 to 8 ml. of plasma containing radioiron. Transfusion was without any untoward symptoms. From 3 to 6 samples of blood with a total volume not exceeding 16 ml. were taken at intervals from 5 to 150 minutes after the transfusion (time after transfusion was counted from the time when half the plasma had been injected). This amount of bleeding was found to be without significant effect on the hematocrit. The blood was added to oxalate and plasma obtained for measurement of radioactivity.

The radioiron was prepared in the Carnegie cyclotron by deuteron bombardment of iron electroplated onto a copper target. It was purified either by the method of Peacock, Evans *et al.* (7) or by repeated extractions with 8N HCl of the ferric chloride dissolved in iso-propyl ether (8). The specific activity, as measured under our standard conditions varied, depending on the sample, from 1.5 to 8 counts per second per microgram. Samples of plasma were prepared for measurement of radioactivity as previously described (8). This consisted essentially of evaporation to dryness in an oven at 100° C. after addition of concentrated HNO₃ and 3 mgm. of carrier iron as ferric nitrate, ashing in a muffle furnace at 450°–500°C., conversion to chloride salts, solution of the ash in saturated ammonium oxalate and electro-deposition of the iron onto a copper disc. The radioactivity was measured with a bell-shaped, self-quenching Geiger-Muller tube with a mica window weighing 3.3 mgm./cm². Results were expressed in terms of microgram of Fe* per ml. plasma by referring each measurement to the radioactivity of a standard disc on which was plated a known quantity of tagged iron. To assure a standard deviation of 1.4 per cent, approximately 10,000 counts were taken on each sample.

RESULTS

The data are presented in table 1. In order to place them on a common basis, as in figure 2, all experiments were adjusted to the same initial concentration of labeled ferric beta₁-globulin in the plasma. The initial concentration in each experiment was obtained by extrapolating the disappearance curve to zero time. This initial concentration, which varied among the 8 animals but averaged about 0.25 microgram Fe* per ml. of plasma, was multiplied by whatever factor was necessary to convert it to a standard concentration of 0.25 microgram Fe* per ml. of plasma. Subsequent plasma concentrations in any one animal were then multiplied by the same factor.

To derive the rate of movement of normally occurring iron out of the capillaries it is assumed that the amount of labeled iron lost from the plasma per unit of time is proportionate to 1) the number of microgram of normally occurring iron which move from plasma to extravascular fluid per unit of time and 2) the proportion of the iron in the plasma which is labeled with radioactive iron. From these assumptions, the change in amount of labeled iron per unit of time is given by:

$$(1) \quad \frac{dFe_p^*}{dt} = -r \frac{Fe_p^*}{Fe_p}$$

where r = microgram of untagged, normally occurring iron which escapes from the capillaries per unit of time, Fe^*_p = microgram of labeled iron in the plasma at any time, t , and Fe_p = microgram of untagged iron intrinsic to the plasma.

TABLE I. CHANGE OF CONCENTRATION OF INTRAVASCULARLY INJECTED, LABELLED FERRIC BETA₁-GLOBULINATE WITH TIME

EXP. NO.	WT. OF ANIMAL	METHOD OF PREP. OF Fe^* -PLASMA	Fe^* -PLASMA TRANSFUSED	TIME AFTER TRANSFUSION	Fe^* /ml. PLASMA	Fe^* /ml. PLASMA ¹	PLASMA VOLUME	
							Found	Anticipated
	gm.		microgm. Fe^*	min.	microgm.	microgm.	ml.	ml.
RP ₄	661	A		10.0	0.65	0.243		
				20.9	0.61	0.228		
				40.5	0.57	0.213		
				49.5	0.56	0.209		
				65.0	0.51	0.190		
				81.5	0.49	0.183		
RP ₅	531	B	22	10.0	0.857	0.248	25	29
				20.0	0.822	0.235		
				30.5	0.790	0.226		
RP ₇	787	A	100	30.3	1.16	0.232	80	34
				50.1	1.05	0.210		
				91.2	1.02	0.205		
				132	0.900	0.180		
				150	0.864	0.174		
RP ₁₀	610	B	5.5	7.0	0.204	0.238	26	31
				15.5	0.182	0.214		
				62.5	0.107	0.119		
RP ₁₁	770	C	5.4	7.0	0.188	0.245	28	38
				30.0	0.168	0.219		
				60.0	0.156	0.203		
RP ₁₂	811	B	16	6.0	0.455	0.244	33	38
				15.5	0.418	0.224		
				40.0	0.350	0.187		
PTP ₁	500	C	7.0	16	0.246	0.234	27	24
				61	0.154	0.146		
				110	0.112	0.106		
PTP ₂	460	C	7.1	24.5	0.330	0.217	19	24
				55.0	0.267	0.176		
				110	0.115	0.076		

A = plasma prepared by addition of Fe^*Cl_3 *in vitro*; B = labelled plasma synthesized by an anemic animal; C = plasma obtained from a donor injected with $Fe^*Cl_3 \cdot Co$ = initial concentration.

The anticipated plasma volume was obtained on the assumption that the average plasma volume = 4.3 ml per 100 gm. body weight (1). Animals PTP 1 and 2 were pregnant.

¹ Adjusted to C_0 (initial concentration) = 0.25 μg .

To express equation 2 in terms of concentration of labeled iron, C^*_p , in the plasma, it is necessary to divide through by the volume of the plasma which gives

$$(2) \quad \frac{dC^*_p}{dt} = -r \frac{C^*_p}{Fe_p}$$

Integrating *equation 1* and solving for the constant of integration by placing $t = 0$ gives

$$(3) \quad \ln C_t^* = -Rt + \ln C_0^*$$

where $R = r/Fe_p$ = the proportion of the iron of the plasma which escapes from the plasma into the extravascular fluid per unit of time and C_0^* is the concentration of labeled iron at $t = 0$.

Equation 3 states that the concentration of labeled iron in the plasma diminishes in such a way that the logarithm of the concentration is a linear function with time. In exponential form *equation 3* may be expressed as

$$C_t^* = C_0 e^{-Rt}$$

which states that the concentration of the tagged material is reduced by a constant proportion per unit of time.

If this treatment is correct, we would expect when the natural logarithm of the concentration of radioiron in any experiment is plotted against time that the points would fall about a straight line the slope of which would be equal to R , the proportion of the iron which escapes from the plasma per unit of time. That this is the case may be seen in figure 2. The accuracy with which the slope can be fixed in a single experiment can be judged from experiments RP7 and RP10 of figure 2. The slopes in 6 of the experiments fell so nearly into 2 groups that they have been presented in this manner in figure 2 for the sake of simplicity. It will be noted that the logarithm to the base 10 of concentration has been used in figure 2 and that R is therefore equal to the slope multiplied by 2.3.

Considerable variation in the rate at which iron is lost from the capillaries is evident among the 8 animals. In the guinea pig with minimal rate, only 0.0025 of that present was lost per minute; in the guinea pig with maximal rate, 0.011 was lost per minute. Six of the 8 animals had rates of loss which varied from 0.0039 to 0.0072 of that present per minute, or expressed in other terms, one half of the iron present at any instant was lost from the plasma in 177 minutes in one group and 96 minutes in the second group. The first of these groups consisted of 3 animals, one of which received by transfusion labeled globulinate synthesized by the anemic guinea pig; one, labeled globulinate prepared *in vitro* and the third, labeled globulinate from a donor injected with Fe^*Cl_3 about 30 minutes before bleeding. In the second of these groups, one animal received by transfusion labeled globulinate synthesized by the anemic animal and the other two labeled globulinate from a donor injected with Fe^*Cl_3 . It is evident from figure 2 that the characteristics of the disappearance curve are independent of the method of preparation of the labeled globulinate.

As can be seen in table 1, plasma volumes calculated from the disappearance curve of tagged ferric globulinate synthesized by the anemic animal or obtained from a donor injected with Fe^*Cl_3 are in the predicted range although, except in one instance, they are lower than the average for the guinea pig found with the dye T-1824 (1).

DISCUSSION

It will be noted that the rates are calculated from data which have been obtained within an interval not exceeding 150 minutes after transfusion. Because of the size

of the animals, it has been difficult to obtain a sufficient number of blood samples to follow further the variation in concentration. It is possible that more than a single rate is involved in the disappearance of radioiron from the plasma and that subsequent rates would not be evident during the time of our observations. It is to be expected, however, that these additional rates, if existent, are relatively slow and would not alter considerably the initial rate of disappearance as we have calculated it for each animal. Evidence confirming the view that these rates at best must be slow has come from measuring the concentration of radioiron in the plasma of 2 guinea pigs 24 hours after transfusion. In these 2 cases the concentration of radioiron was within the limits predicted from extrapolation of the extremes of the initial rates given in figure 2.

The contrast between the permeability of the capillaries to sodium and water on the one hand, and iron on the other is a striking one. In the guinea pig, 60 per cent of the sodium and 146 per cent of the water of the plasma is exchanged each minute with extravascular sodium and water. Only 0.3 to 1 per cent of the ferric globulinate is lost from the plasma per minute. If the capillaries were equally permeable to all these substances, their rates of turnover in the plasma would be identical. The results permit us to say that the capillaries are at least 100 times as permeable to water as to iron.

As stated in the introduction to this paper, it is not certain that iron crosses the capillary wall as the beta₁-globulinate but if this assumption is made the results are consistent with current concepts. The difference in permeability to water and ferric globulinate is as would be expected from their difference in mass. The finding that the capillary wall is at least 100 times as permeable to water as to iron would mean in terms of this assumption that fluid passing through the capillary wall contains one per cent or less of the ferric globulinate of the plasma and fits Landis' (9) statement "that the capillary endothelium can, and often does, retain 95 per cent of the plasma protein." We are currently endeavoring to put this assumption to further test.

The disappearance curve of intravenously injected tagged sodium and D₂O is characterized by the establishment of an equilibrium concentration in the plasma approximately 10 minutes after the injection. The disappearance curve of labeled ferric globulinate, however, up to a period of 150 minutes after intravascular injection shows no indication of return of labeled iron from the extravascular fluid to the plasma. This is equivalent to saying that iron lost from the plasma is returned relatively slowly and is not remarkable if it is supposed that extravascular iron combines with apoferritin and that iron must be split from ferritin before reappearing in the plasma.

SUMMARY

With ferric beta₁-globulinate tagged with radioactive iron as the tracer material, the rate of transcapillary exchange of iron has been determined in the guinea pig. Only from 0.3 to 1 per cent of the iron of the plasma is exchanged per minute. On the assumption that iron crosses the capillary wall as ferric beta₁-globulinate, it can be said that the capillaries are at least 100 times more permeable to water than to the globulinate.

It appears that tagged ferric globulinate prepared by intravascular injection of Fe^*Cl_3 into a donor animal followed by exsanguination 30 minutes later is acceptable for measurement of plasma volume in laboratory animals.

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VASOCONSTRICTION AND THE DEVELOPMENT OF IRREVERSIBLE HEMORRHAGIC SHOCK¹

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NATURE'S provision of a sympathogenic mechanism for rapidly inducing generalized vasoconstriction in the event of a severe hemorrhage is common knowledge. That the latter greatly reduces the circulatory capacity, and simultaneously redistributes considerable volumes of blood from so-called 'luxury' organs to those structures whose function is vital for immediate survival, is fully recognized. This *initial* beneficial influence of vasoconstriction permits greater loss of blood without the development of hemodynamic crises.

The realization of the possibility that continuance of severe vasoconstriction in addition to the arterial hypotension incident to hemorrhage may prove 'deleterious' is much less commonly expressed in the literature. Yet there is considerable indirect evidence available to support this contention. That vasoconstriction increases in severity as *impending*³ shock progresses towards irreversibility through elaboration of humoral vasoconstrictor agents is seen from the reports of Page *et al.* (3, 4) and Zweifach *et al.* (5). This would certainly tend to further aggravate the blood supply to large areas which had already been severely reduced by low arterial pressures and reflex vasoconstriction. Introduction of pressor drugs during a severe hemorrhagic-hypotension (6-8), thus inducing even greater vasoconstriction in the 'luxury' structures, seems to accelerate the transition from *impending* to *irreversible* shock in spite of temporary elevations of blood pressure in the reduced circulation. Over a decade ago, the reports of Freeman *et al.* (9, 10) not only substantiated the significance of the 'initial beneficial influences' of vasoconstriction but also suggested the 'secondary detrimental influences' of prolonged vasoconstriction. Further indirect evidence of the possible harmful effects of protracted vasoconstriction was accumulated by these investigators (11) while reproducing the clinical syndrome of shock with continuous infusion of adrenalin.

It seemed advisable, therefore, to investigate further the influence of vasoconstriction upon the course of events in a hemorrhagic procedure standardized to induce an irreversible shock state within a 90-minute experimental period. The plan was

Received for publication April 26, 1948.

¹ This work was initiated under a grant from the Graduate School Research Board of the University of Illinois. It was completed with a grant-in-aid from the U. S. Public Health Service.

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³ The *impending hemorrhagic shock* state may be said to prevail whenever the residual blood volume following cessation of an uncomplicated hemorrhage is less than 60% of the normal pre-hemorrhagic blood volume.

to compare the survival rates of a series of control animals with those of another group deprived of their sympathogenic vasoconstrictor mechanisms. It was concluded that if the survival rates were significantly greater among the latter group, it would definitely indicate the deleterious influence of protracted vasoconstriction. The advent of the adrenergic blocking agent, dibenamine hydrochloride (12), provided a means of blocking sympathogenic vasoconstrictor mechanisms of humoral origin and of reducing, if not completely blocking, those of reflex origin, without the necessity of recourse to surgical sympathectomy and its undesirable features.

EXPERIMENTAL PROCEDURES

Though the procedures for inducing the *impending* and *irreversible hemorrhagic shock* states were essentially those standardized previously in this laboratory (11), it has been sufficiently modified to require brief description.

Femoral arteries and veins were isolated and cannulated under local procaine hydrochloride anesthesia. Following the customary period for control observations, the dogs were bled rapidly (5-7 mgm/kgm/min.) into a graduated cylinder containing adequate quantities of the anticoagulant, Liquaemin.⁴ This was terminated when the desired hypotensive level was attained. The latter level was constantly maintained for 90 minutes thereafter by means of an automatic pressure stabilizing device. In this way the more laborious and less accurate manual techniques used in previous investigations were obviated. A calibrated reservoir bottle, partially filled with some of the withdrawn heparinized blood, was connected to the side arm of a femoral arterial cannula. This reservoir was then elevated to the level required to maintain the established hypotension level. Thus, any tendency for the arterial pressure to rise above 40-43 mm. Hg was abruptly arrested by automatic bleeding from the dog into this reservoir. Contrarily, any tendency for arterial pressure to decline below the established level was prevented by an immediate automatic infusion of withdrawn blood from the reservoir into the animal. At the end of the 90-minute hypotension period, all remaining withdrawn blood was reinfused via a femoral vein. The operative sites were then sprinkled with sulfanilamide powder and sutured. Upon return to their quarters, the animals were permitted limited quantities of water only for the next 18 hours. Those animals which were alive on the 4th post-operative day were considered to have recovered completely from the *impending shock* state which had been induced. The remainder revealed the typical symptoms and post-mortem changes of *irreversible shock*.

RESULTS AND DISCUSSION

A. Prior to describing the experimental observations, the terminology employed will be clarified.

1. That volume of blood which is rapidly withdrawn during the initial bleeding process to establish the prescribed degree of arterial hypotension is referred to as the *initial bleeding volume* (IBV). The factors determining the magnitude of the IBV are several. Assuming a constant rapid rate of hemorrhage and a certain level of hypotension at which point the *rapid* withdrawal of blood is terminated, there are still the compensatory factors to be considered. Theoretically, an animal devoid of any compensatory mechanisms would yield a certain basic bleeding volume before the established hypotension level is attained. This basic bleeding volume will be augmented in the control animal, depending upon the rate and extent to which his

⁴ We are greatly indebted to Roche-Organon, Inc. for their generosity and cooperation in supplying us with the quantities of the anti-coagulant Liquaemin required for the execution of this study.

compensatory adjustments can be mobilized. The most rapidly induced adjustments appear to be those under reflexogenic vasomotor control, such as reduction of the total circulatory capacity and expression of blood into the circulation from various blood depots. Of secondary importance in the augmentation of the IBV is the slowly mobilized process of auto-infusion of the circulation from the extra-vascular fluids. Indication of the secondary nature of this process in the production of the IBV is seen in the very slight reduction of IBV values obtained in poorly hydrated dogs. It would seem, therefore, that the increase in IBV over basic values is primarily a function of the extent of reflexogenically controlled vasomotor compensation.

2. Near the middle of the hypotension period, the *maximal bleeding volume* (MBV) was usually reached. This constituted the IBV plus that volume which automatically entered the pressure-stabilizing reservoir in the process of maintaining blood pressure at a constant hypotensive level. In the few control dogs which eventually survived the *impending shock* state induced by the procedure (i.e., following reinfusion of all withdrawn blood at the termination of the 90-minute hemorrhagic-hypotension period), the MBV was frequently attained towards the very end of the hypotension period. In the majority of control animals, however, considerable volumes of blood were automatically reinfused to sustain the desired hypotensive level during the last 45 minutes of the hypotension period.

3. The total quantity of blood available for reinfusion as the 90-minute period terminated is referred to as the *net-total bleeding volume* (N-TBV). It obviously represents the MBV minus the quantity automatically reinfused, if any, to sustain the established hypotensive level. The prognosis for complete recovery among untreated control dogs was excellent whenever the MBV and N-TBV volumes were essentially equivalent, i.e., when the automatic reinfusion volume (ARV) was negligible or very small (the ARV is the MBV minus the N-TBV). Conversely a reasonably satisfactory diagnosis of prevailing *irreversible* shock can be made in control dogs when the ARV is greater than 4.0 ml/kgm. It will be seen that such diagnoses are not possible in the dibenamine-treated dogs. This automatic reinfusion is undoubtedly initiated by the same forces which were responsible for the persistent spontaneous decline in arterial blood pressure observed in previous experiments which antedated the introduction of the automatic pressure stabilization device (2). The latter decline was one of several criteria found to be reliably diagnostic for the early onset of *irreversible* shock.

4. The volume of blood automatically withdrawn after the hypotension level was attained (MBV minus the IBV) is referred to as the *secondary bleeding volume* (SBV). Its magnitude seems to be primarily determined by the animal's ability to mobilize body fluids to augment the existing circulating blood volume. The SBV is markedly reduced in dehydrated animals which have much less fluid available for auto-infusion purposes. Thus the SBV provides a reliable index of the animal's ability to mobilize fluids for augmentation of the circulating blood volume, even though vasomotor reactions play a secondary rôle in determining its magnitude.

Twenty-four dogs were subjected to 90 minutes of hypotension at 40-43 mm. Hg, of which 12 served as untreated controls. The remaining 12 dogs received an intravenous injection of dibenamine (10-15 mgm/kgm.) 30 minutes prior to the onset

of bleeding procedures. It was believed that a functional blocking of all sympathogenic vasoconstrictor mechanisms had been induced by the dibenamine in each instance. The basis for this belief is as follows: *a*) an adrenaline reversal was obtained in each animal before bleeding was begun and *b*) moments of excitement or struggling in these dogs were accompanied by a significant decline rather than an elevation of blood pressure.

The most significant data obtained from these 24 animals are included in table 1. The somewhat high recovery rate (41.7 per cent) attained in these 12 control dogs was surprising and disappointing, since it was significantly greater than the 23.1 per cent recovery observed in a previous control series, in which procedures were identical with the exception that manual bleeding techniques were employed (11). It is difficult to conceive that this difference in survival rate may be related to the adoption of the automatic bleeding and reinfusion device. A more likely influence might

TABLE 1

SERIES	NO. OF DOGS	% RECOVERY		MEDIAN VALUES FOR BLEEDING VOLUMES IN ML/KGM.				
				IBV	MBV	N-TBV	SBV	ARV
Controls	12	41.7	T	34.9	47.5	42.3	11.8	6.0
	5		R	35.1	55.3	52.5	15.8	2.6
	7		F	34.9	45.6	36.4	10.2	6.8
Dogs given dibenamine 30 min. before hemorrhage	12	25.0	T	19.4	19.8	10.7	2.2	9.1
	3		R	18.4	20.2	14.3	2.5	5.9
	9		F	19.7	19.7	7.6	0.0	14.9

T—Values for entire series, including fatalities and recoveries. R—Values for recovery dogs. F—Values for fatality dogs. IBV—Initial bleeding volume—described in text. MBV—Maximal bleeding volume—described in text. N-TBV—Net-total bleeding volume—described in text. SBV—Secondary bleeding volume—described in text. ARV—Automatic reinfusion volume—described in text.

be the difference in the state of nutrition and hydration of the dogs at the time of experimentation. Regardless of this discrepancy, the recovery rate in these 12 control dogs is useful and adequate for comparison with that observed in the dogs treated with dibenamine.

As seen in table 1, the recovery rate in the dibenamine-treated dogs (25%) was even lower than in the untreated controls (41.7%). It is possible, from some of the experimental observations, to offer at least a partial explanation for the poorer response among these treated dogs. Twenty minutes after the injection of dibenamine, an 'adrenalin reversal' was elicited in all animals. It is therefore presumed that any sympathogenic vasomotor tone existing prior to the introduction of the drug, as well as the mechanisms for compensatory augmentation of peripheral vasoconstriction during the hemorrhagic period which followed, were functionally blocked. *A priori*, the total circulatory capacity was undoubtedly greatly enlarged prior to the onset of hemorrhage, thus accounting for the exceptionally small bleeding volumes obtained

during the ensuing hemorrhagic-hypotension period. Despite the apparent increase in total circulatory capacity, however, the control blood pressures remained essentially unaltered. One would be inclined to attribute the sustained blood pressure level to a compensatory increase in plasma volume as a result of autotransfusion of tissue fluids, were it not for the fact that the hematocrits also remained unaltered. To utilize this explanation it would be necessary to assume an equivalent discharge of erythrocytes from various blood depots. The mechanism, however, for such emptying of cellular depots is not apparent, since dibenamine appears to block the mechanisms for splenic contraction (14). One is therefore inclined to attribute the maintenance of normal blood pressure to some influence which augments cardiac output. This is suggested by the pronounced tachycardia seen in all these dogs (median 180 beats/min.). Whatever the responsible factor or factors, they are apparently only temporarily adequate to sustain normal pressure levels, inasmuch as more recently conducted experiments (17) reveal a significant decline in arterial blood pressure within a 10- to 12-hour period following the administration of dibenamine. This decline is then more slowly compensated. Although not thoroughly appreciated at the time these experiments were conducted, it is now obvious that the cardiovascular conditions, in the dogs treated with dibenamine just prior to hemorrhage, were definitely unstable since neither the effects of the drug nor the mobilization of compensatory adjustments had fully materialized.

The exceptionally small IBV in this series of treated dogs reflects the value of sympathogenic vasoconstrictor mechanisms as an immediate adjustment for a rapid loss of blood. It also suggests that the dibenamine in the doses given blocked the nervous as well as the humoral components involved in this normal compensatory adjustment. Comparatively, these animals seemed less able to withstand hemorrhage than the sympathectomized dogs reported by Freeman (9). This difference may be related to the fact that Freeman's animals were permitted a reasonable interval following sympathectomy during which more effective circulatory adjustments for the loss of sympathogenic vasomotor activity could take place.

A comparison of the respective bleeding volumes for the control and the treated animals reveals striking differences (table 1). In the control group the 5 *complete recoveries* (R) exhibited a significant SBV and in each the MBV and N-TBV values were nearly equivalent. Hence, ARV values were negligible or small. In the 3 *recovery* dogs of the dibenamine group, however, the IBV and MBV values were nearly equivalent. Consequently the SBV was exceptionally small or absent. The median ARV value in these dogs was about twice that seen in the control recoveries, indicating that considerable automatic reinfusion was required to sustain the hypotensive level during the latter portion of the hypotension period. Comparing the ARV of these two groups, in terms of the percentage of the MBV automatically reinfused, these differences are even more striking. Whereas 5 per cent of the MBV was reinfused in the control recovery dogs, 29.2 per cent automatic reinfusion occurred in the dibenamine recovery dogs, even though their MBV values were roughly one-third those obtained from the control dogs. From this aspect it is indeed surprising that these 3 dibenamine animals did not enter the *irreversible* shock state, inasmuch as this course of events has in past experiences been commensurate with very short post-

reinfusion survival times. It is possible that the cause underlying the sizeable reinfusions seen in the dibenamine recovery dogs may be related to progressive action of the drug itself whereas in the fatality dogs of the control series, they were initiated by widespread disturbances of the vasomotor mechanisms and/or capillary permeability.

The absence of any significant SBV in the treated group is puzzling. Presumably, conditions should have been extremely favorable for translocation of tissue fluids into the vascular compartment. In the group of dogs 'pre-treated' with dibenamine 20 hours in advance of the onset of hemorrhagic procedures (to be discussed below), the influx of tissue fluids under what appears to have been essentially equivalent hemodynamic conditions was much greater, as indicated in the SBV values (table 2). One can only speculate that a similar compensatory augmentation of the circulating plasma volume was in some manner impeded by an as yet unrecognized influence of dibenamine.

TABLE 2

SERIES	NO. OF DOGS	% RECOVERY		MEDIAN VALUES FOR BLEEDING VOLUMES IN ML/KGM.				
				IBV	MBV	N-TBV	SBV	ARV
Controls	20	30	T	35.0	48.0	42.3	12.0	5.9
	6		R	35.2	55.5	52.8	15.9	2.7
	14		F	35.0	45.7	36.5	10.2	6.8
Dogs given dibenamine 20 hr. before hemorrhage	20	60	T	27.6	35.9	30.4	6.6	3.0
	12		R	28.0	42.3	41.1	8.2	0.3
	8		F	24.1	31.6	16.5	6.2	12.1

For explanation of abbreviations see table 1.

In recapitulation, these preliminary studies seem to emphasize the importance of immediate adjustment of the circulatory capacity through participation of sympathogenic vasomotor mechanisms as a compensation for severe hemorrhages. They fail, despite original hopes, to provide pertinent information that would permit practical evaluation of the degree of compensation which can be enlisted more gradually through accessory mechanisms when sympathogenic vasoconstriction is temporarily eliminated. Furthermore, this series of investigations provides no justification for drawing conclusions regarding the effect of prolonged vasoconstriction upon the transition from *impending* to *irreversible* shock.

B. The initial alteration in procedure in this next series of experiments involved an increase in the severity of the hemorrhagic process to guarantee the induction of *irreversible* shock in a higher percentage of untreated control animals than was accomplished in the previous series. It was apparent that this might be managed either by prolonging the hypotension period or by increasing the severity of the established hypotension level. The latter approach was selected and the hypotensive level was lowered to 35-38 mm. Hg. That this more nearly approached the ideal effect is reflected in the 30 per cent survival rate among the 20 untreated control dogs

(table 2) as compared with the 41.7 per cent survival rate in the previous series of control dogs. There were 2 dogs which succumbed about 72 hours following reinfusion of all withdrawn blood. These bore a striking resemblance to certain 'border-line' cases described previously (2). They were arbitrarily included among the 6 survivals, their deaths being attributed to secondary causes. If *shock* was the cause of death in these 2 animals, the survival rate would have amounted to 20 per cent.

As in earlier studies, relative and occasionally actual hemoconcentration developed during the 90-minute hypotension period in each animal. It appears, however, that the development of hemoconcentration bore a definite relation to the automatic reinfusion which occurred in order to check the tendency for blood pressure to fall below the established hypotensive level. As in the previous series (table 1), the bleeding volumes among the 14 control *fatality* dogs (*F*, table 2) were significantly smaller than in the 6 *complete recoveries* (*R*, table 2). The implications are obvious and require no further discussion.

It was also necessary to alter procedures to allow sufficient time prior to the onset of hemorrhaging for the dibenamine to attain its maximal effect, as well as for the mobilization of adequate compensatory adjustments for the hemodynamic disturbances resulting from the actions of this agent. With the latter in mind, 20 dogs were 'pre-treated' with an intravenous injection of dibenamine (15-20 mgm/kgm. doses) some 20 hours prior to initial bleedings.

The results of this approach were strikingly different in that 14, or 60 per cent, of these 'pre-treated' dogs recovered completely from the 90-minute exposure to hemorrhagic hypotension at 35-38 mm. Hg. This improvement in survival rate gains particular significance in view *a*) of the greater severity of the hypotension in this series as indicated by the low survival rate (30%) among control dogs and *b*) of the poor survival rate (25%) in the previous group of dibenamine treated dogs.

In an attempt to ascertain the basis of the more favorable responses in this 'pre-treated' group, it is found that analysis of the immediate pre-hemorrhagic status of these 'pre-treated' dogs, as well as the course of events during hypotension, affords only a partial explanation. Immediately prior to the onset of hemorrhage and 20 hours after the administration of dibenamine, the existing tachycardia was equivalent in magnitude to that in the previous dibenamine group. In this respect alone were they similar. In distinct contrast, arterial blood pressure levels approached the lower limits (ca. 100 mm. Hg) customarily accepted as normal for a group of untrained mongrel dogs. In fact, these control levels were considerably lower than the mean value of control measurements obtained in over 250 animals in earlier studies (ca. 129 mm. Hg). Furthermore, the control pre-hemorrhagic hematocrits were consistently 10 or more units lower than those encountered in previous control measurements on untreated dogs. *A priori*, it is not difficult to visualize the course of cardiovascular events which followed the administration of dibenamine. As compensation for an expanded circulatory capacity, ensuing the functional blocking of sympathogenic vasoconstrictor mechanisms and the consequent progressive decline of arterial blood pressure, it is probable that a large auto-infusion of tissue fluid into the vascular compartment occurred. A similar mobilization of fluids and a resultant augmentation in plasma volume (up to 25%) was reported by Gregersen *et al.* to have occurred

after surgical sympathectomy (14). Since an 'adrenalin reversal', as well as the decline in blood pressure concomitant with excitement or struggling, was clearly demonstrated in most of these dogs some 20 hours after the injection of dibenamine, it is hardly conceivable that any sympathogenic vasoconstrictor mechanisms were still functional at this time. In this respect, these 'pre-treated' dogs were still functionally sympathectomized. By the end of this 20-hour control period, arterial pressures, though perhaps normal, had not reached their pre-dibenamine levels.

Unlike the situation in the earlier dibenamine series, there was no tendency for arterial pressures to decline progressively throughout the hypotension period, due to increasing effectiveness of the drug. It seems reasonable, furthermore, to assume that under the prevailing conditions of generalized vascular dilatation 20 hours after treatment with dibenamine, the existing (though slightly subnormal) control blood pressure levels were sufficient to provide adequate peripheral blood flow. In fact these 'pre-treated' dogs gave every evidence, just prior to hemorrhage, that their cardiovascular conditions were far more stable than those in the group which received dibenamine only 30 minutes preceding the onset of hemorrhage.

Comparing the data obtained in the treated and pre-treated series during the 90-minute hemorrhagic-hypotension period, the following observations are made. *a)* The IBV values for the 'pre-treated' dogs (table 2) are considerably greater than those for the treated group (table 1), although they are not as large as in the control group. The larger IBV values in the control as compared to the 'pre-treated' animals is attributed to the reflex induction of sympathogenic vasoconstriction which was presumably unavailable to the latter group of animals. *b)* The SBV values for the 'pre-treated' recovery dogs (table 2, *R*) were sizable and significant, especially when compared with similar values among the 'treated' recoveries (table 1, *R*). It would seem that the difference between the SBV values in the control and dibenamine 'pre-treated' recovery dogs is an indication of the part which progressive sympathogenic vasoconstriction contributes toward the SBV. It must be remembered, however, that considerable auto-infusion had occurred in these animals during the 20-hour control period following administration of the dibenamine and that the latter undoubtedly restricted the amount of fluid available for auto-infusion during hemorrhagic hypotension. In other words, this may, to a great extent, account for the difference between the SBV of the control and 'pre-treated' animals.

It is obvious that the onset of *irreversibility* occurred (during the 90-minute hypotension period) less frequently among the 'pre-treated' (dibenamine) dogs than in their respective controls. The question arises as to how dibenamine influenced the rate of transition from *impending* to *irreversible* shock. Since the SBV values in many fatality dogs of the untreated control groups were not greatly reduced, there must be additional responsible influences other than those which determine the magnitude of the SBV. In animals whose sympathogenic vasoconstrictor mechanisms have been functionally blocked, the major impairment in tissue blood flow must be the low arterial pressure. It is reasonable to assume, however, that, per given blood pressure level, the overall tissue blood flow will be greater in the animals with subnormal vasoconstrictor tone than in control dogs in which greater resistance to arterial flow is created through vasoconstriction of reflex or humoral sympathogenic origin. It is

not surprising to find an increasing number of reports in the literature citing evidence of extensive morphological and function damage of such organs as the kidney, liver, and intestines following the induction of severe hemorrhagic conditions. It is possible that the irreversible phase of shock often first becomes apparent when the function of one or more of these organs becomes so greatly impaired as to be incommensurate with complete recovery. Certainly the ingenious studies by Frank *et al.* (15) substantiate this consideration. These investigators reveal that the high incidence of *irreversible* shock in untreated control dogs, induced by hemorrhagic procedures quite similar to those cited here, can be reversed to a high incidence of recovery when the liver is viviperfused by a donor animal during the course of hemorrhagic-hypotension. Perhaps the slight improvement in intestinal, kidney, and liver blood flow afforded at 35–38 mm. Hg blood pressure levels, by the dilatation of vessels through the actions of dibenamine, was just sufficient to prevent the occurrence of extensive irreversible damage in these organs during the experimental period. One would not, of course, expect the same degree of vasodilation in all animals following uniform dibenamine treatment; hence, the transition to irreversible shock was not sufficiently delayed in some animals of this series. In this way, the improved survival rate of the 'pre-treated' dogs might be partially explained. From these studies, the conclusion is drawn that the prolonged vasoconstriction induced by severe hemorrhage, when superimposed upon an already restricted peripheral blood flow due to arterial hypotension, greatly accelerates the transition from *impending* to *irreversible* hemorrhagic shock. Presumably this is related to permanent derangements of such 'luxury' organs as the intestinal tract, the liver and the kidney to an extent which is incompatible with life.

SUMMARY

1. In untreated dogs, the blood flow through such organs as the kidney, liver and intestines during the *impending hemorrhagic state* may be progressively impaired by a) arterial hypotension, b) superimposed vasoconstriction of sympathogenic origin and possibly by c) additional vasoconstriction through liberation and action of humoral agents, the origin and classification of which is not clearly established.

2. To determine the extent to which prolonged vasoconstriction and the consequent protracted impairment of visceral blood flow may influence the rate of transition from *impending* to *irreversible* shock, the adrenergic blocking agent, dibenamine, was employed to eliminate factor b and perhaps c above. Its influence upon the survival rate in standardized hemorrhagic shock producing experiments was then analyzed.

3. Initially, the dibenamine was administered to dogs only 30 minutes before hemorrhagic procedures were begun. The progressive influence of the drug for several hours after its administration rendered the cardiovascular conditions at the onset of bleeding extremely unstable. Hence, the original purpose of these experiments was not accomplished. It was felt that the use of the drug in this manner merely rendered the animal's compensatory adjustments much less efficient.

4. Another group of dogs were then 'pre-treated' with dibenamine 20 hours in advance. This permitted full attainment of drug action and nearly complete mobili-

zation of compensatory adjustments for the cardiovascular influences of this drug. Hence, greater cardiovascular stability prior to the onset of bleeding was achieved. Evidence is provided that at the time of bleeding, sympathogenic vasoconstrictor mechanisms were still effectively blocked in all 'pre-treated' dogs. As a consequence of this pretreatment, the transition from *impending* to *irreversible* shock was delayed in many instances to the extent that the incidence of *irreversible* shock was greatly reduced when compared to such incidence in an equivalent number of control untreated animals.

5. From the results, it appears that even though initial vasoconstriction offers temporary benefits, by augmenting the amount of blood which can be lost before critical changes in blood pressure occur, there can be little doubt that the prolonged continuance of sympathogenic vasoconstriction is deleterious in that it accelerates the onset of the *irreversible* state.

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OXYGEN SATURATION IN BONE MARROW, AND IN ARTERIAL AND VENOUS BLOOD DURING PROLONGED HEMORRHAGIC ERYTHROPOIESIS¹

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FOLLOWING hemorrhage, the normal red blood cell mass is soon restored by increased erythropoietic activity of the bone marrow. It is generally believed that qualitatively similar erythropoiesis is in progress at all times in the normal adult and serves to replace the small quantity of erythrocytes which are daily destroyed. On the other hand, it is possible to elicit an abnormal increase in the red blood cell mass by a variety of means both experimental and pathological. The red bone marrow participates, therefore, in the maintenance of a normal, constant hemoglobin concentration as well as in the elevation of this pigment far above normal values. Solely on the basis of the oft observed 'polycythemic response', usually associated with reduced arterial O₂ saturation, (altitude, emphysema, etc.) it has been maintained that a low O₂ saturation of the blood *in bone marrow* is the primary stimulus for all erythropoiesis. This explanation was probably first advanced by Miescher in 1893 (1) and although untested has been widely accepted.

Grant and Root (2) examined this hypothesis by studying the O₂ saturation of blood drawn directly from red bone marrow of unanesthetized dogs. Erythropoiesis was induced by a single, large hemorrhage. A low bone marrow blood O₂ saturation was evident for only a few hours following hemorrhage, after which normal values were found throughout the three or more weeks required for the regeneration of lost red blood cells. The present study has the same purpose. Instead of a single hemorrhage, the animals were rendered and maintained anemic by frequent, small hemorrhages. The erythropoietic stimulus produced in this manner was both more intense and uniform over a relatively long period of time. If a low O₂ saturation of bone marrow blood is required to initiate the production of red blood cells, blood drawn directly from the red bone marrow of these animals should show a low O₂ saturation.

METHODS

Animals. Eight adult male dogs with body weights ranging from 6 to 11.5 kgm. were used in this study. The animals were fed dog meal (Beacon Milling Company) mixed with water. An average daily ration contained from 50 to 60 mgm. total iron. On this regimen the hemorrhaged animals were maintained in good condition and showed a moderate hemoglobin production which remained constant throughout the period.

Analyses. O₂ content was measured by the Roughton-Scholander (3) micro-method on 0.04 cc. of blood and O₂ capacity determined by a modification of this

Received for publication May 28, 1948.

¹ A preliminary report of this work appeared in *Federation Proc.* 7: 43, 1948.

method previously described (4). Both O₂ content and O₂ capacity were determined in all blood samples for which the percentage O₂ saturation is given. Hemoglobin concentration was calculated from the O₂ capacity, and Van Allen hematocrit tubes were used to estimate the relative volume of packed red blood cells. The methods for the measurement of reticulocyte percentage and plasma volume were the same as those used in a previous study (2).

Sampling. All blood samples were taken from unanesthetized dogs, which had been without food for 18 to 24 hours and had been resting quietly in a supine position for at least 15 minutes. The technique of removing blood from red bone marrow of the proximal end of the humerus has been described previously (2). By use of the micro-methods, percentage O₂ saturation as well as hematocrit value were measured in a sample as small as 0.1 cc. of blood. All analyses of bone marrow blood were performed on the first 0.1 to 0.2 cc. to be drawn from the marrow. No blood samples were drawn for 24 hours following hemorrhage.

TABLE 1. AVERAGE O₂ CAPACITY LEVEL IN CONTROL AND ANEMIC PERIODS AND DAILY HEMOGLOBIN PRODUCTION IN THE ANEMIC PERIOD

DOG	WT.	O ₂ CAPACITY		Hb/DAY
		Control	Anemia	
				gms.
1	8.7	19.0	11.0	3.3
2	8.0	15.5	10.0	3.0
3	11.0	17.5	10.5	4.2
4	8.2	14.5	9.0	3.2
5	11.5	14.7	9.5	4.0
6	9.5	18.0	10.0	3.5
7	6.0	22.0	10.5	2.0
8	8.0	15.5	11.0	2.7

Maintenance of Anemia. Anemia was induced and hemoglobin regeneration measured in the manner described by Whipple and Robscheit-Robbins (5). Their now classical method was not rigidly followed because of certain differences in the purpose of these experiments.

At the end of the control period, dogs were bled every two or three days until the jugular venous O₂ capacity values ranged between 9 and 11 volumes per cent (6.7 to 8.2 grams Hb.). This level represents 48 to 71 per cent (average 61) of the control O₂ capacity values of the 8 dogs (table 1). Blood was drawn by syringe through an 18-gauge needle inserted in the femoral artery. (Arterial puncture has been performed some 25 times in a single artery with no difficulty.) O₂ capacity was maintained at a fairly constant level (± 1.5 vol. per cent in the individual dog) by additional small hemorrhages every two to three days. The volume of blood to be removed in each instance was determined by the degree to which the O₂ capacity exceeded the desired level. Hemoglobin production was calculated on the basis of drawn blood (5). In order to avoid erroneous information from O₂ capacity values in the event of temporary hemodilution or concentration, plasma volume was occa-

sionally estimated. Under the conditions of the experiments, the plasma volume in the anemic animal remained relatively stable. The anemic period varied from 40 to 100 days.

The formation of red blood cells was indicated by *a*) hemoglobin production, *b*) increased percentage of reticulocytes and other immature cells and *c*) decreased saturation index (O_2 capacity \times 2.2/hematocrit percentage).

PROCEDURE

The O_2 saturation of arterial, jugular venous and bone marrow blood was measured once or twice during the control period. No determinations of this type were performed in the period of 8 to 14 days during which the hemoglobin concentration was being reduced by hemorrhage to a predetermined anemic level. When a relatively constant level had been established, O_2 saturations of bone and venous blood were measured at approximately weekly intervals, while arterial blood was examined less frequently. The majority of these analyses were performed two days after a hemorrhage. At the end of the hemorrhagic period, the hemoglobin concentration in the blood was permitted to rise to control value; and in several of the dogs, O_2 saturation was again determined.

RESULTS

Hemoglobin Production. Since the object of this study was erythropoiesis it was essential to employ a quantitative measure of the process. Table 1 contains a summary of the hemoglobin regeneration rates. The average daily production for all dogs was 3.2 grams Hb or about 22 grams Hb per week. The dogs employed by Whipple and Robscheit-Robbins (5) had normal O_2 capacity values (calculated by the present author) of 22 to 27 volume per cent and were bled to anemic levels of approximately 7.5 to 10.0 volume per cent (calculated). The O_2 capacity of dogs at the beginning of the present experiments ranged from 14.5 to 22 volume per cent and were reduced to a level of 9 to 11 volume per cent. The anemia induced by the Rochester group was, therefore, more severe. In addition, the present animals received a stock diet of dog meal which on analysis was shown to provide 50 to 60 mgm. of total iron per day. At no time was an attempt made to fortify this basic ration with extra quantities of such potent hemoglobin-forming agents as liver, beef muscle etc. Despite certain differences in procedure, regimen and body weight, the rates of hemoglobin regeneration shown by the animals in this study compare favorably with those reported by the Rochester group.

In 5 of the 8 animals an apparent over-production of hemoglobin occurred following the conclusion of hemorrhage. The O_2 capacity values rose from the anemic level to exceed that of the original control period. This 'overshoot' has been observed by others and is probably a result of either *a*) increased erythropoietic efficiency or *b*) hyperplasia of red bone marrow, or both (6).

Typical reticulocytosis occurred in all animals a few days after the first hemorrhages and persisted throughout the period. With the 'dry smear' technique, between 2 and 5 per cent of red blood cells displayed a stained reticulum. Occasional films were prepared with blood drawn by the routine bone marrow puncture. Blood

so obtained was identical with that used in the measurement of O_2 saturation. By inspection, it was evident that the number of immature erythroid cells had greatly increased in the marrow.

The saturation index during the control period varied between 0.95 and 1.05. The value decreased in the anemic phase to a range of 0.80 to 0.90. Following the cessation of hemorrhage, the saturation index increased with the O_2 capacity values to approach control values.

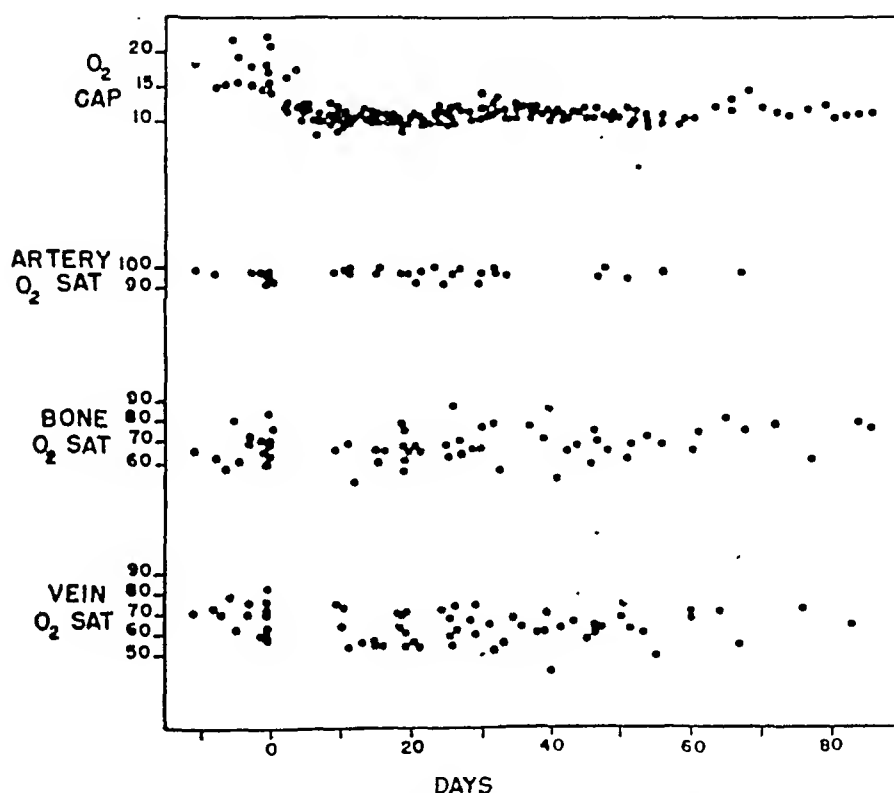


Fig. 1. A COMPOSITE OF THE RESULTS obtained from 8 unanesthetized dogs before and during the anemia produced by repeated hemorrhages. The onset (zero day) and severity of anemia can be determined from the O_2 capacity values. It will be noted that the O_2 saturation of arterial femoral and bone marrow (humerus) blood show the same level and distribution in the control and anemic periods. Jugular venous O_2 saturation is slightly depressed in anemia.

Oxygen Saturation. A) The percentage O_2 saturation of femoral arterial blood was unaffected by the hemorrhagic anemia. Values obtained in both control and anemic periods displayed remarkable constancy and no significant difference was noted. A composite graph (fig. 1) containing the results from all dogs shows the arterial O_2 saturation in relation to the O_2 capacity in the normal and hemorrhagic periods. Table 2 presents a summary of blood gas data.

B) In marked contrast to the relative constancy of O_2 saturation values of arterial blood, those obtained from *bone marrow* blood revealed a wide range of individual variation. This is true for the normal as well as for the anemic animals.

In 5 out of the 8 dogs, no anemic O_2 saturation percentage was found below that

of the control period. The remaining 3 animals occasionally showed a temporary O₂ saturation which was 2 to 10 per cent below control values. In the one animal in which this phenomenon was pronounced, it was noted that when the O₂ saturation of the bone marrow blood reached its nadir, hemoglobin production ceased temporarily. At this point it was necessary to omit the usual hemorrhages and before proceeding with the experiment to permit a partial recovery of both O₂ capacity and O₂ saturation. This cycle was repeated in the same animal on two further occasions with essentially the same results.

Considering the 8 dogs as a group, no significant differences can be shown between values obtained in the control and those in the anemic period. As presented in table 2, the average results from all 8 animals is 68.5 per cent O₂ saturation for the normal and 68.3 for the anemic dog.

C) The range of O₂ saturation percentage found in *jugular venous* blood was comparable to that encountered in the bone marrow. There was a tendency, however, for the venous anemic values to fall slightly below those obtained in the control period. This is shown graphically in figure 1 and numerically in table 2. The

TABLE 2. PERCENTAGE O₂ SATURATION OF ARTERIAL (FEMORAL), BONE MARROW (HUMERUS) AND VENOUS (JUGULAR) BLOOD IN CONTROL AND ANEMIC PERIODS

O ₂ SAT	CONTROL			ANEMIC		
	No.	Range	Ave.	No.	Range	Ave.
Artery.....	8	93-99	96.0	19	90-100	96.4
Bone.....	13	57-85	68.5	42	51-87	68.3
Vein.....	14	59-83	69.5	44	46-74	64.8

grand average from 8 animals gives a control value of 69.5 per cent O₂ saturation and 64.8 in the anemic phase.

Arterial-Venous Difference. The difference between the O₂ content of femoral arterial and jugular venous blood (A-V) and between that of the same artery and marrow blood (A-M) were less in the anemic period as compared with the control. In figure 2, A-V and A-M values from 8 dogs are plotted against the corresponding O₂ capacity, and it will be noted that a rough but *direct* relationship exists. On the contrary, an *inverse* relationship was found between A-V and A-M values and the percentage O₂ saturation of the corresponding venous and bone marrow blood. This relationship is shown in figure 3 and includes results from both control and anemic periods.

DISCUSSION

Erythropoietic Activity. Under the conditions of this study, all reservoirs of preformed erythrocytes must have been depleted in the first few weeks of hemorrhage. Whether untapped depots of the precursors of hemoglobin and red cell stroma exist beyond this interval is of secondary importance in this experiment. The diet was constant throughout the entire period and adequate in regard to hemoglobin production as the evidence indicates. No attempt was made to fortify it with

extra liver, muscle or iron. The issue here is one of the primary stimulus to the formation and delivery to the general circulation of mature erythrocytes rather than a study of dietary factors.

As a result of repeated hemorrhages, the red bone marrow responded by a marked increase in erythropoietic activity. As an indication of the magnitude of the production of red blood cells, consider the following example. In table 1, *dog 4* had an average weight of 8.2 kgm. and a weekly hemoglobin production rate of 22 grams. This amount of hemoglobin is equivalent to 76 cc. of packed red blood cells at the ratio provided by the existing saturation index. Fairman and Whipple (7) have ingeniously measured the volume of the bone marrow space in the dog and found it to correspond to approximately 2.4 per cent of body weight, with marrow being assigned a specific gravity of unity. *Dog 4*, therefore, would have a marrow space or volume of about 200 cc., part of which undoubtedly contains inactive tissue. Thus, in one week an organ with a volume of 200 cc. produces 76 cc. of red blood cells or 38 per cent of its own maximum volume. In view of this rather remarkable growth rate, it may be safely stated that active erythropoiesis was in progress in the red bone marrow.

Compensation in Hemorrhagic Anemia. Under the conditions of this study O_2 capacity was controlled by regular, measured bleedings. It has been noted that the A-M and A-V O_2 differences vary directly with the O_2 capacity, and further that the O_2 saturations of marrow and venous blood vary inversely with the corresponding A-M and A-V values (fig. 2 and 3). This compensatory decrease in A-M and A-V differences observed in the anemic condition is probably a result of an increased cardiac output. A marked increase in cardiac output was reported by Blalock and Harrison (8) in dogs rendered anemic by repeated hemorrhage. In clinical investigations a moderate augmentation of cardiac output has been noted in the severely anemic patients (9) as well as an increased peripheral blood flow (10).

From these studies, it is reasonable to assume that the decrease in A-M and A-V values was a result of an increased cardiac output. Reduction in the O_2 consumption of tissues generally is not necessary and it has also been demonstrated that no significant decrease in O_2 consumption occurs in the anemia secondary to chronic hemorrhage (8, 11).

As judged by the relative constancy of the O_2 saturation of bone marrow blood and to a lesser degree that of the jugular venous blood following repeated hemorrhages, circulatory compensation was generally good. The observation that bone blood O_2 saturation showed no significant change from control to anemic period while jugular venous blood displayed a small decrease in O_2 saturation may possibly be explained by vasodilation in the marrow with an increased blood flow. Another factor may be the difference in O_2 requirements of the areas drained by the two types of blood.

The animals were reduced to an average of 61 per cent of their control hemoglobin levels. Compensation was efficient with this degree of anemia. It would have been possible to have reduced further the hemoglobin concentration and thus to have rendered the compensatory mechanisms inadequate. Also, it must be recalled that these animals led a sedentary and confined life and were examined at rest usually 48

hours after hemorrhage. The anemia level employed in this work was therefore adequate to stimulate active erythropoiesis, yet not severe enough to overwhelm the compensatory adjustments.

Erythropoiesis and Anoxia. That chronic anoxic anoxia (low arterial pO₂) induces polycythemia is well known. The mechanism by which the bone marrow is stimulated under this condition is open, however, to speculation. The present results indicate that it is possible to cause a tremendous increase in erythropoiesis while maintaining a normal O₂ saturation in the blood of red bone marrow. It is apparent that under conditions of general anoxic anoxia the marrow is also anoxic. These

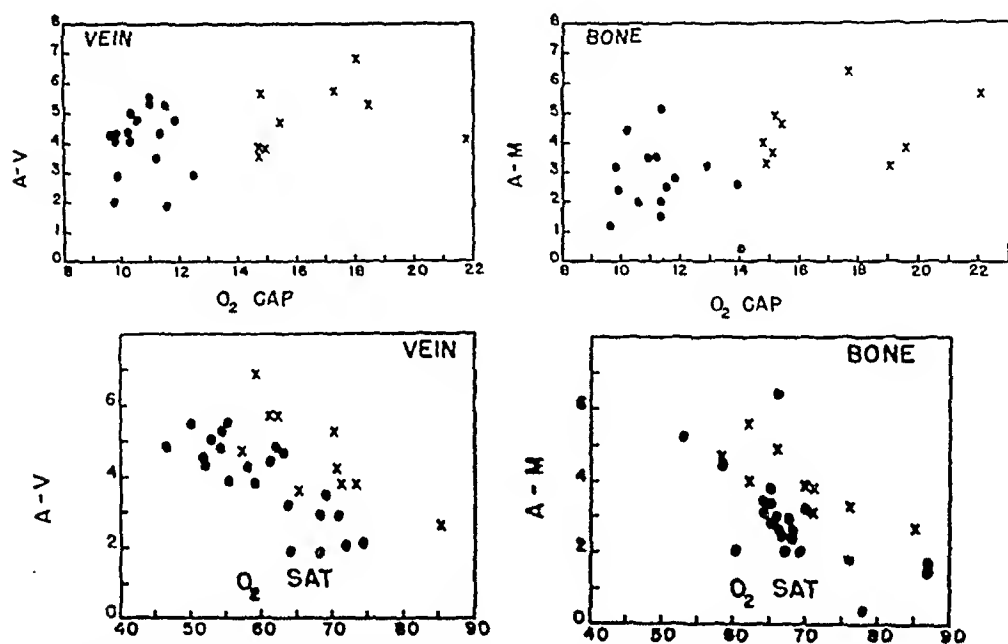


Fig. 2. *Upper.* THE DIFFERENCE between the O₂ content of femoral arterial and jugular venous blood (A-V) and between that of the same artery and marrow blood (A-M) are plotted against the corresponding O₂ capacity. The results are obtained from 8 dogs in both control and anemic periods. A rough but direct relationship is shown between the two functions. The A-V and A-M values of the control period (X) tend to exceed those of the anemic (dot).

Fig. 3. *Lower.* A-V AND A-M VALUES (for description see fig. 2) plotted against the O₂ saturation of jugular venous and marrow blood respectively. The results are obtained from 8 dogs and both control (X) and anemic (dot) period values are given. An inverse relationship exists between the A-V and A-M values and the appropriate O₂ saturation.

observations suggest that a) two mechanisms for the stimulation of erythropoiesis exist or b) the relationship between the low O₂ saturation of marrow and erythropoiesis is fortuitous or of only secondary importance. A recent study by Rosin and Rachmilewitz (12) is pertinent. The growth of marrow cells maintained in tissue culture ceased when exposed to an O₂ tension of 21 mm. Hg, was moderate at 100 mm. Hg and accelerated at 357 mm. Hg. Absolute pO₂ values under these unphysiological conditions are questionable. More important are the relative values which indicate that marrow cell growth is stimulated by an increase in pO₂ and not, as has been supposed, by a decrease.

SUMMARY

Anemia was induced in 8 male dogs by repeated small hemorrhages and this condition was maintained for 40 to 100 days. The anemia level was determined from the O_2 capacity, which was reduced to approximately 60 per cent of the control value. Stimulation of erythropoiesis was demonstrated by the daily production of hemoglobin and reticulocytosis.

Percentage O_2 saturation of blood obtained from the red bone marrow of the unanesthetized animals and that from the femoral artery and jugular vein was measured before and during the anemia period. No significant difference was evident in the O_2 saturation of bone marrow blood in the anemia period when compared with that in the control period. Arterial blood showed the same constancy in O_2 saturation, but that of jugular venous blood decreased slightly in anemia. The evidence indicates that a striking and prolonged stimulation of erythropoiesis occurred even though the O_2 saturation of bone marrow blood remained at normal levels.

Certain aspects of the circulatory compensation in anemia are discussed with reference to arterial-marrow O_2 content difference.

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NATURE OF 'UNIPOLAR' EXTREMITY LEADS IN THE DOG.¹

LEAD VR

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IN THE anesthetized dog the 'unipolar' extremity lead VR may normally exhibit one of several patterns (fig. 1). These patterns closely resemble those found in man. The currently accepted explanation of the derivation of the ventricular complex in lead VR is based upon a number of assumptions. *a)* The complex usually resembles that obtained by leading directly from the right ventricular cavity. Therefore lead VR is said to reflect mainly the potential variations of this cavity (1, 2). *b)* The exploring electrode on the right forelimb or shoulder is said to lie opposite the valvular orifices at the base of the heart, thus permitting it to 'look' into the ventricular cavity and reflect mainly its potential variations (1). *c)* Variations in potential of the ventricular cavity are said to develop from the sequential appearance and disappearance of local differences in potential between the endocardial and epicardial surfaces of the heart (2, 3). *d)* When the left side of the interventricular septum is activated prior to the right side, it is said that the spread of excitation through the septum from left to right can be detected by the unipolar electrocardiogram (1-3). *e)* Differences in onset and rate of repolarization of the endocardial and epicardial surfaces of the heart may be responsible for the direction of the T-wave (4, 5).

Based upon these assumptions the following explanation has been given for the ventricular complex in lead VR. The excitatory process usually first reaches the left side of the septum, thus rendering the right ventricular cavity electropositive until the excitation wave has spread through the thickness of the septum to the right side. This early positivity of the right cavity causes the upstroke of the small R-wave frequently seen in lead VR and in leads obtained directly from the right cavity. When the excitation process reaches the endocardium of the right ventricle, the right cavity becomes electronegative with respect to the epicardial surface, thus resulting in the beginning downstroke of the R-wave. As the excitation wave continues to travel outward from endocardium to epicardium in both ventricles, the cavities of both ventricles continue to remain electronegative and the beam continues to move downward, inscribing the downstroke of the large S-wave. When the epicardial surfaces are finally depolarized, no difference in potential exists between the endocardial and epicardial surfaces and the beam returns to the isopotential line, inscribing the upstroke of S. The ST-segment when isopotential is the period when the whole heart is depolarized and no currents are flowing. The explanation suggested for a normally inverted T-wave in lead VR is that the epicardial layers of the heart complete their repolarization before the endocardial layers and thereby render the cavity electronegative until the process of repolarization is complete in the entire heart.

Received for publication April 16, 1948.

¹ Aided by grants from the Fluid Research Funds, Yale University School of Medicine and the U. S. Public Health Service.

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Experiments of Hoff and Nahum have already cast doubt on the validity of some of the above assumptions, for they have found that the spread of the excitation process from the endocardial to the epicardial surface of the heart cannot be detected in unipolar precordial or in standard limb lead electrocardiograms (6, 7). Furthermore, altering the rate of repolarization of either epicardial or underlying endocardial areas of the ventricles did not produce opposite effects on the direction of the T-wave (8). These investigators, therefore, were led to an alternative explanation for the origin of the ventricular complex in 'unipolar' precordial leads. They found that a region of the heart 'proximal' to the exploring electrode, when activated in any part of its thickness, caused downward movement of the beam in the electrocardiogram. Potentials resulting from excitation in regions 'distal' to the exploring electrode caused upward movements of the beam when either the endocardial or epicardial surfaces in these 'distal' regions were activated. They concluded that the position of the beam at any moment in the ventricular complex was determined by the algebraic summation of all simultaneous 'proximal' and 'distal' effects.

The following experiments which were undertaken to study the derivation of lead VR in the dog lend support to the 'zonal interference' theory of Nahum and Hoff (9). Subsequent communications will deal with a similar experimental study of the derivation of leads VL and VF.

METHODS

Thirty dogs were employed in these studies. The animals were deeply anesthetized with Dial and the heart was exposed as described previously (10). All electrocardiographic tracings were taken with a Sanborn Tribeam only after the lungs were fully inflated, the chest wall tightly closed by means of skin clips and spontaneous respiration present. These precautions are necessary since it has been demonstrated (11) that removal or displacement of electrically conducting tissues from their normal relation to the heart results in alterations of the electrocardiographic complex. Oxygen was constantly administered by endotracheal catheter. All electrodes used in recording lead VR were inserted subcutaneously. The indifferent electrode consisted of a central terminal which was connected by insulated copper wires of equal length to the right and left forelimbs and to the left hindlimb. Lead VR was recorded by pairing an exploring electrode on the right forelimb with this indifferent electrode. Connections to the electrocardiograph were such that relative negativity at the exploring electrode resulted in downward movement of the beam, while relative positivity at the exploring electrode resulted in an upward movement of the beam.

RESULTS

Impressed Potentials

Method. Pairs of needle electrodes were inserted through the intact pericardium in such a way that they maintained contact with the epicardium without injuring it. The electrodes were connected by fine insulated copper wires to a potentiometer across one dry cell. A commutator in the circuit made it possible to change the polarity at the electrodes. Potential differences of low voltage were applied momentarily across these two electrodes at intervals and the resultant beam deflections recorded in lead VR. Hoff, Nahum and Kaufman (12), utilizing this method in a study of standard limb leads, found that in the electrocardiogram the distribution of potentials physically applied at the surface of the heart followed closely that of biopotentials generated by the heart itself. This method of impressed potentials permits a rough delimitation of those regions of the heart surface which, when made negative with

respect to the rest of the heart, result in a downward movement of the beam in lead VR (proximal zone), and those regions which, when made negative with respect to the rest of the heart, result in an upward movement of the beam in VR (distal zone).

When one electrode was placed on various points of the anterior or posterior surface of the right ventricle, and the other on various points on the anterior or posterior surface of the left ventricle, impressed electronegativity on the surface of the right ventricle and electropositivity on the surface of the left ventricle caused a downward movement of the beam in lead VR (fig. 2A). Reversal of polarity so that the

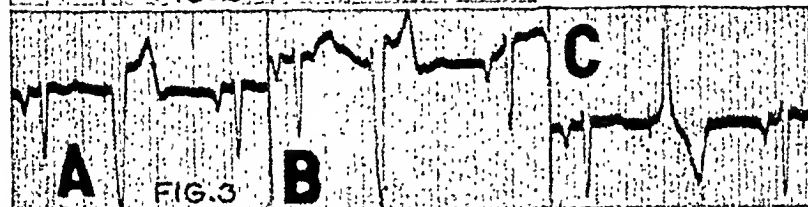
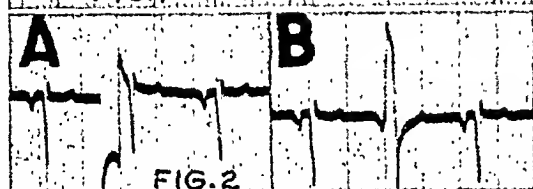
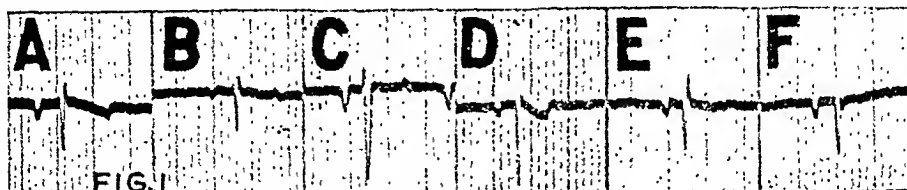


Fig. 1. A, B, C, D, E AND F ARE VARIOUS NORMAL PATTERNS seen in dogs under Dial anaesthesia, before the chest is opened. There are variations in the QRS complex and also in the direction of the T-wave.

Fig. 2. DOG, 4.1 KGM., FEMALE, 2/17/48. One electrode on surface right lateral ventricle and another electrode on surface left lateral ventricle. A, impressed negativity at electrode on right ventricle; the beam moves downward. B, impressed negativity at electrode on left ventricle; the beam moves upward.

Fig. 3. PATTERNS OF EXTRASYSTOLES elicited by stimulation of the epicardial surface of the various segments of the heart. Dog, 6.0 kgm., female, 10/28/47. A, extrasystoles elicited from anterior and posterior right ventricle (except for right apex), upper third anterior septum, and from basal portion anterior left ventricle near the septum. Initial movement of beam is downward. B, extrasystoles elicited from stimulation of lower two thirds of the anterior septum, parts of the right apex, the posterior septum and the basal portion of the anterior left ventricle. Small initial upward deflection followed by downward deflection. C, extrasystoles elicited from stimulation of the left apex, lower two thirds of the anterior left ventricle and posterior left ventricle. Initial movement of the beam is upward.

electrode at the surface of the right ventricle was electropositive resulted in an upward movement of the beam (fig. 2B).

Forced Epicardial Extrasystoles

Method. After opening the pericardium, bipolar needle electrodes were inserted into the epicardium. Intermittent break shocks were delivered approximately every second by means of a thyatron stimulator, the stimulus strength being just above threshold. An occasional extrasystole was thus elicited against a background of normal complexes.

Exploration of the anterior and posterior surfaces of both ventricles yielded three different types of extrasystoles.

Type A (fig. 3A). The ventricular complex exhibits a QS deflection and an up-right T-wave. This type of extrasystole could be obtained only by stimulation of points on the anterior and posterior surface of the right ventricle (except for the right apex) and from a small segment of the basal portion of the anterior left ventricle near the septum.

Type B (fig. 3C). The ventricular complex exhibits a large R-wave followed by an inverted T-wave. This type of extrasystole could be obtained only by stimulation of points on the anterior and posterior surface of the left ventricle including the left apex, except for a small segment of the basal portion of the anterior left ventricle near the septum.

Type C (fig. 3B). The ventricular complex exhibits an rS or qRs complex, usually followed by an upright T-wave. This type of extrasystole could be obtained only by stimulation of points on the anterior and posterior surface of the right apex, from the anterior and posterior septal areas and from a small segment of the basal portion of the anterior left ventricle.

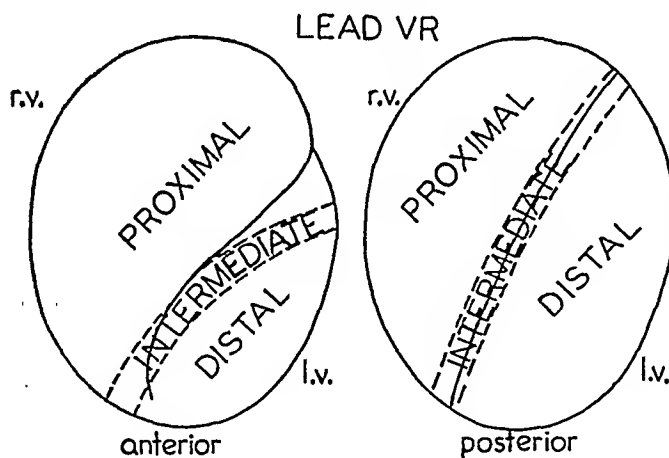


Fig. 4. SCHEMATIC DRAWING of the anterior and posterior surfaces of the dog heart outlining the location and extent of the proximal, intermediate and distal zones of lead VR.

The heart surface is, therefore, divided into three distinct zones from which extrasystoles of each of the above described types may be elicited (fig. 4). Zone A, from which extrasystoles of the QS pattern are obtained, is designated as the *proximal* zone of lead VR, since it consists of that portion of the heart surface lying closest to or 'facing' the exploring electrode on the right forelimb. Zone B, from which extrasystoles of the R pattern are obtained, is designated as the *distal* zone of lead VR, since it consists of that portion of the heart surface lying furthest from or 'facing away' from the exploring electrode. Zone C, from which extrasystoles of the rS or qRs pattern are obtained, is designated as the *intermediate* zone of lead VR, since it consists of that portion of the heart surface which separates the proximal from the distal zones.

Thus, when depolarization is experimentally initiated in any portion of the proximal zone, the initial movement of the beam is downward. Conversely, when depolarization is initiated in any portion of the distal zone, the initial movement of the beam is upward. When depolarization is initiated in the intermediate zone, the direc-

tion of beam movement is variable. It may initially be deflected downward if the site of stimulation is adjacent to the proximal zone; upward, if the site of stimulation is adjacent to the distal zone; or the beam may remain stationary if the site of stimulation is equidistant from the two zones. From these observations it can be inferred that downward movement of the beam at any moment during the inscription of the QRS complex is due to preponderance of depolarization in regions of the heart which constitute the proximal zone, while an upward movement of the beam is due to preponderance of depolarization in regions of the heart which constitute the distal zone.

Effect upon ST-segment of Surface Injury from Application of M/5 KCl Solution or Superficial Cautery

Method. Squares of filter paper soaked in M/5 KCl solution were applied to various segments of the heart surface according to the method of Kisch, Nahum and Hoff (10). After each application the filter paper was removed, the heart and pericardium were thoroughly washed with warm Ringer-Locke solution and time was allowed to permit the electrocardiogram to return to normal before treating a new area. Superficial burns were produced by cauterizing the surface of the heart. It has already been shown that such surface injury produces an injury current which in turn causes a persistent displacement of the diastolic baseline of the electrocardiogram (13). The direction of displacement of the diastolic baseline will depend upon the relation of the exploring electrode to the injured region. When the exploring electrode 'faces' the injured region it is in the field of negative potential, and the diastolic baseline will be displaced downward and result in an 'elevation' of the ST-segment. When the exploring electrode 'faces away' from the injured region, it is in the field of positive potential, and the diastolic baseline will be displaced upward and result in a 'depression' of the ST-segment.

Application of KCl to various segments of the heart surface as well as superficial burning yielded one of three different effects:

ST-segment elevation. This is obtained following treatment of the anterior and posterior surface of the right ventricle (except the right apex), and of the basal portion of the anterior left ventricle near the septum (fig. 5A).

ST-segment depression. This is obtained following treatment of the left apex, the posterior left ventricle and the lower two thirds of the anterior left ventricle (fig. 5C).

No ST-segment displacement or minimal displacement up or down. This is obtained following treatment of the right apex and the anterior and posterior septal regions (fig. 5B).

This experimental method permits determination of the areas of the heart which are so spatially oriented to the exploring electrode that when injured ST-segment elevation will result, and those areas which are so oriented to the exploring electrode that when injured ST-segment depression will result. It can be seen that the areas of the heart which when injured result in ST-segment elevation are the same areas which have been found to constitute the proximal zone by the method of impressed potentials and forced epicardial extrasystoles. Similarly, the areas of the heart which when injured result in ST-segment depression are the same areas which have been found to constitute the distal zone.

Immediate Effects of Coronary Ligation on ST-segment

Method. Individual coronary vessels were isolated by fine dissection and the artery and its accompanying veins were occluded by means of a silk ligature or a metal clamp. Successful occlusion of the vessels resulted in the prompt development of purplish discoloration of the heart wall supplied by the particular vessels. Electrocardiograms were taken at frequent intervals after occlusion.

The following observations were made after coronary ligation:

Occlusion of right coronary artery. This is usually followed by ischemia of the upper half of the anterolateral wall of the right ventricle. Lead VR exhibits elevation of the ST-segment (fig. 6A).

Occlusion of posterior descending branch of right coronary. This is usually followed by ischemia of a portion of the posterior right ventricle. Lead VR exhibits an elevation of the ST-segment (fig. 6B).

Occlusion of anterior descending branch of left coronary. This is usually followed by ischemia of the anterior septal area, but the extent of ischemia varies in different animals because of anatomical variations in vessel distribution and of differences in the relative adequacy of collateral circulation. Lead VR may exhibit elevation, depression or no change in ST-segment (fig. 6C, D and E).

Occlusion of lateral descending branch of left coronary. This usually produces ischemia of the anterolateral wall of the left ventricle. Lead VR exhibits depression of the ST-segment (fig. 6F).

Occlusion of posterior descending branch of left coronary. This usually causes ischemia of the posterior left ventricle and the posterior left apex. Lead VR exhibits depression of the ST-segment (fig. 6G).

Injury occurring in areas of the heart which lie in the proximal zone of lead VR, therefore, causes elevation of the ST-segment, while depression of the ST-segment occurs when injury is limited to areas of the heart lying in the distal zone. If an injury is so situated as to involve areas of the heart which lie both in the proximal and the distal zones, as in the case of anterior septal infarcts, no ST-segment displacement may occur, or the direction of the displacement will vary, depending upon the relative extent of the infarction in each zone.

No attempt has been made to correlate the changes of the T-wave occurring immediately after coronary artery ligation, since it is now known (14) that the drop in temperature at the surface of the heart on merely opening the chest wall is by itself sufficient to produce a striking effect on the configuration and direction of the T-wave. Only after the chest has been closed for several hours will the environmental temperature influences be eliminated and permit an analysis of the T-wave in relation to the factor of ischemia.

Effect upon T-wave of Heat and Cold on Epicardial Surface

Method. A flat circular metal chamber 2.8 cm. in diameter with inlet and outlet tubes was inserted through a slit in the pericardium and placed upon the epicardial surface of the heart. Control records were taken with the chamber in place. Then water at temperature of 50°C. was continuously circulated through the chamber

while electrocardiographic records were being taken. A change in the temperature of the particular segment of the heart surface being thus treated was recorded by

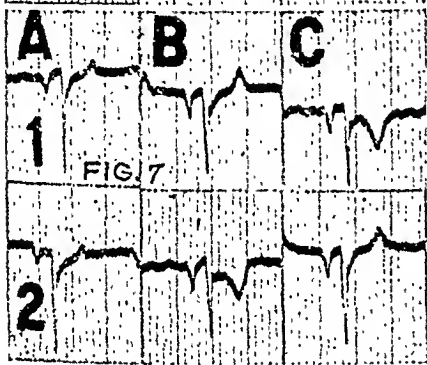
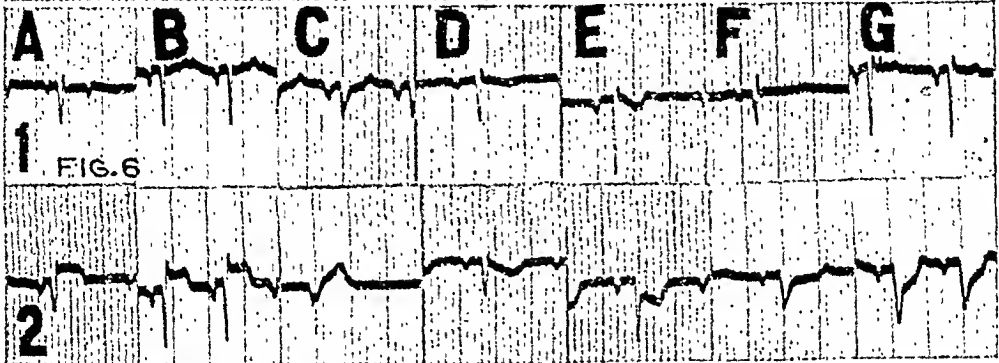
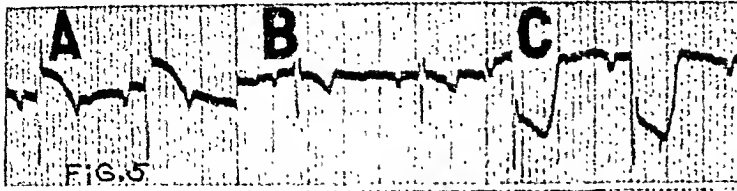


Fig. 5. ST-SEGMENT DEVIATIONS following application of M/5 KCl to various parts of the heart surface. Dog, 9.0 kgm., male, 7/31/47. A, proximal zone, ST-segment elevated. B, intermediate zone, ST-segment isopotential. C, distal zone, ST-segment depressed.

Fig. 6. ST-SEGMENT DEVIATIONS following occlusion of various coronary vessels. 1, control; 2, following ligation. A, ligation right coronary artery and vein. Dog, 7.5 kgm., male, 1/6/48. ST-segment elevated. B, ligation right circumflex vessels. Dog, 4.1 kgm., female, 2/17/48. ST-segment elevated. C, ligation anterior descending branch left coronary artery. Dog, 8.7 kgm., male, 8/7/47. ST-segment slightly elevated. D, ligation anterior descending branch left coronary artery. Dog, 6.6 kgm., male, 9/2/47. ST-segment slightly depressed. E, ligation anterior descending branch left coronary artery. Dog, 9/11/47. ST-segment depressed. F, ligation lateral branch left coronary artery. Dog, 8.4 kgm., female. ST-segment depressed. G, ligation posterior descending branch left coronary artery. Dog (same as in B). ST-segment depressed.

Fig. 7. EFFECT OF HEATING AND COOLING proximal and distal zones on the direction of the T-wave. Dog, 7.4 kgm., male, 9-4-47. 1, chamber on proximal zone; 2, chamber on distal zone. A, control. B, water at 50°C. circulating through chamber. Heating proximal zone, T-wave more upright; heating distal zone, T-wave inverted. C, water at 20°C. circulating through chamber. Cooling proximal zone inverts T-wave. Cooling distal zone makes T more upright.

means of copper-constantan thermocouples fixed in position in the immediately overlying pericardium. After each procedure time was allowed to permit the temperature of the treated surface and the electrocardiographic changes to return to the con-

trol normal range. Water at 15 to 20°C. was then circulated through the chamber and records again taken. During the heating and cooling of any particular region of the heart, simultaneous temperature readings were taken from several other regions of the heart by means of thermocouples inserted in the overlying pericardium in order to be certain that the temperature change had occurred only in the regions under study. This method permits the study of the effect upon the T-wave of accelerating and decelerating the repolarization process in the various zones.

When repolarization of the proximal zone is accelerated by heating the anterior or posterior epicardial surface of the right ventricle, the T-wave becomes more positive. When repolarization of this zone is slowed by cooling it, the T-wave becomes more negative. When repolarization of the distal zone is accelerated by heating the epicardial surface of the left ventricle, anterior or posterior, the T-wave becomes more negative. Conversely, delaying the repolarization of the distal zone by cooling it results in a more positive T-wave (fig. 7).

DISCUSSION

Electrocardiograms recorded in the manner described in these experiments have been designated as unipolar, since the potential of the indifferent electrode is considered to remain constant and approximate zero (1). The resulting beam movements are therefore considered to reflect solely variations in potential at the site of the exploring electrode. That the central terminal may indeed vary from zero has been demonstrated by Wolferth (15) and others (16). Leads taken in this manner, therefore, are not truly unipolar but are so-called for convenience inasmuch as they tend to approximate unipolarity. Despite these considerations, the above experiments demonstrate that in such a lead the direction of beam movement following depolarization in various segments of the heart depends solely upon the spatial orientation of the depolarized areas to the exploring electrode.

The effect of depolarization in any part of the areas of the anterior and posterior right ventricle (except the right apex), the upper third of the anterior septum and a small portion of the upper anterior left ventricle near the septum (the *proximal* zone) is to move the beam downward in lead VR. The effect of depolarization in any part of the areas of the anterior and posterior left apex, the posterior left ventricle and the lower two thirds of the anterior left ventricle (the *distal* zone) is to move the beam upward.

The position of the beam at any given moment in the ventricular complex will be determined by the algebraic summation of the potentials arising from depolarization at that moment of regions of the heart lying in the proximal zone which tend to move the beam downward, and potentials arising from depolarization of regions lying in the distal zone which tend to move the beam upward. When the beam is deflected downward it indicates preponderance of electrical activity due to depolarization during that moment in the proximal zone. Conversely, an upward movement of the beam denotes preponderance of electrical activity in the distal zone. If at any moment the algebraic summation of the potentials derived from depolarization in both zones is zero, the beam will remain stationary. If this occurs at the onset of the ventricular complex, the beam will remain at the isoelectric line even though excita-

tion of the heart is in progress. An isoelectric interval may, therefore, result from one or more of several possibilities: *a*) when the entire heart is depolarized and no currents are flowing; *b*) when initial or terminal depolarization is occurring in equal degree in both proximal and distal zones; *c*) when initial or terminal depolarization is taking place in the intermediate zone; *d*) when repolarization is proceeding in equal degree in both proximal and distal zones; and *e*) when repolarization is completed everywhere. In order to determine which of these possibilities is in operation in a given case it is necessary to obtain simultaneous recording of two or more 'unipolar' leads.

If during any short interval the algebraic summation of simultaneous potentials arising from proximal and distal zones gives rise to a constant value, the beam will remain stationary during that interval, inscribing a 'plateau' in the ventricular complex. The position of the beam at any given moment in the T-complex is also determined by the algebraic summation of potentials derived from the various heart zones during the repolarization process. When repolarization is proceeding more slowly in the distal zone than in the proximal zone, the proximal zone is electrically relatively positive with respect to the distal zone and an initial upward movement of the beam will occur, inscribing an upright T-wave. Conversely, initial downward movement of the beam will occur when repolarization proceeds more slowly in the proximal zone than in the distal zone and an inverted T-wave will therefore be inscribed. Thus, one can infer from the direction of the T-wave the order of repolarization of the proximal and distal zones of lead VR.

This knowledge of the nature of beam movements in lead VR gained from the present experiments permits an analysis of the normal ventricular complexes seen in this lead in the dog. The initial small R-wave, when present, indicates that a part of the distal zone is the first to receive the excitation process. The quick reversal of the beam downward indicates rapid spread of excitation to a part of the proximal zone. For this sequence to occur the first region of excitation in the distal zone must lie adjacent to the proximal zone (the anterior or posterior left ventricle near the septum). The continued downward movement of the beam inscribing the downstroke of *S* signifies a preponderant spread of excitation to involve portions of the proximal zone (the right ventricle). The upstroke of *S* indicates later spread of excitation to involve the remaining portions of the distal zone (the major part of the left ventricle). When an R'-wave is present the downstroke indicates that the last region of the heart to be excited lies in the proximal zone. By other methods (17) it has been demonstrated that the right ventricle near the conus may be the last part of the heart to become depolarized. More specific localization of the spread of excitation in the dog heart can be derived from a study of three 'unipolar' extremity leads, VR, VL and VF, and one or two precordial leads recorded simultaneously, and this will be discussed in a subsequent communication. The ST-segment in lead VR is usually isoelectric. From an analysis of lead VR alone, it is not possible to state which one of, or combination of, the possible causes of isoelectric ST-segments discussed above may be responsible.

When the T-wave is upright in lead VR, it indicates that repolarization begins and terminates in the proximal zone in advance of the distal zone. Since the major

portion of the proximal zone is depolarized in advance of the distal zone, its earlier repolarization might be expected. However, when the T-wave is normally found to be inverted, indicating a later repolarization of the proximal zone despite its earlier depolarization, the explanation for this is not yet apparent.

The experiments herein described render untenable the hypothesis that variations in the potential of the ventricular cavity are mainly responsible for the formation of the ventricular complex in lead VR for the following reasons: *a*) Forced extrasystoles from a major portion of the epicardial surface of the anterior and posterior right ventricle and from the basal portion of the anterior left ventricle near the septum show no initial R-wave, although according to the cavity potential hypothesis such an R-wave is to be expected since the right ventricular cavity would be electro-positive until the excitation process has spread through to involve the endocardial surface. Pallares *et al.* (18) on mechanical stimulation of the canine right ventricular surface also found that extrasystoles thus elicited exhibited no initial R-wave, but only a QS complex. *b*) When injury of the epicardial surface of the right ventricle or the basal portion of the anterior left ventricle near the septum is produced by the application of M/5 KCl or by superficial burning with cautery, the ST-segment is elevated, although it should be depressed if cavity potentials determine the nature of lead VR, since the injury is limited to the surface of the heart and the cavity would be in the electropositive field. *c*) If the direction of the T-wave is to be explained on the theory that the endocardial surface repolarizes later than the epicardial surface, thus rendering the cavity initially electronegative and the T-wave therefore inverted, heating the epicardial surface, thus further accelerating its repolarization, should render the T-wave more negative. However, the opposite occurs when the epicardium of the right ventricle is heated, the T-wave markedly increasing in positivity. On the other hand, delaying the repolarization at the epicardial surface of the right ventricle by cooling it should produce an upright T-wave, whereas these experiments reveal that the T-wave is invariably made more negative. That portion of the thicker anterior left ventricle which lies in the proximal zone of lead VR shows effects identical with those obtained from the thinner right ventricle.

SUMMARY

1. The ventricular complex in lead VR is inscribed as a result of interference between electrical events due to depolarization in a region of the heart 'facing' the electrode (the proximal zone), which cause downward movement of the beam, and electrical events occurring in a region 'facing away' from the exploring electrode (the distal zone), which cause upward movement of the beam.

2. For lead VR the proximal zone consists of the anterior and posterior right ventricle (except for the right apex), the upper third of the anterior septum and a portion of the upper anterior left ventricle near the septum. The distal zone consists of the anterior and posterior left apex, the posterior left ventricle and the lower two thirds of the anterior left ventricle. There is an intermediate zone separating the proximal and distal zones which consists of the anterior and posterior right apex, the anterior and posterior septal areas and a small portion of the basal anterior left ventricle.

3. The spread of excitation and recovery in the various parts of the dog heart have been roughly charted by analyzing the position of the beam at successive moments in the ventricular complex.

4. Injury to the heart can be localized by study of ST-segment deviation. Elevation of the ST-segment indicates existence of injury in the proximal zone, while depression of the ST-segment denotes injury in the distal zone.

5. Localization of the site of origin of ventricular extrasystoles may be determined from their configuration. Those that arise in the proximal zone exhibit a QS complex; those that arise in the distal zone exhibit an R-complex; while those that arise in the intermediate zone exhibit an rS or qRs complex.

6. The order of repolarization in the heart can be determined from the configuration of the T-wave. An upright T-wave results when that portion of the left ventricle which lies in the distal zone repolarizes more slowly than the right ventricle. Conversely, an inverted T-wave develops when the right ventricle repolarizes more slowly than the left ventricle.

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NATURE OF 'UNIPOLAR' EXTREMITY LEADS IN THE DOG.¹ LEAD VL

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IN THE dog, the 'unipolar' extremity lead VL may normally exhibit one of several patterns (fig. 1). These patterns resemble those found in man when the heart is considered to be in a 'vertical' position (1). The currently accepted explanation for the derivation of this complex is based upon the assumption that in a heart which lies in a 'vertical' position the exploring electrode situated upon the left forelimb 'looks' into the cavity of the left ventricle and thus mainly reflects the variations in potential of this cavity during the cardiac cycle (1). The results already obtained from the study of the nature of lead VR (2) have brought into serious question the explanation that because an electrode 'faces' a cavity of the heart the electrocardiogram recorded by it reflects mainly the changing potential of that cavity.

In the following experiments, methods similar to those employed in the study of lead VR were used to determine the derivation of lead VL. It was found that lead VL is derived from an interference between instantaneous electrical events during the cardiac cycle occurring in regions of the heart designated as proximal, intermediate and distal with respect to the position of the exploring electrode. Lead VL was recorded by pairing an exploring electrode inserted subcutaneously in the left forelimb with a central terminal electrode constructed as described previously (2).

Impressed Potentials

When one electrode was placed on any point on the upper two-thirds of the anterior surface of the heart and the other electrode on the apex or posterior surface of the heart, negativity of the electrode on the anterior surface caused a downward movement of the beam in lead VL (fig. 2A). Reversal of polarity so that the electrode on the upper anterior surface was positive resulted in an upward movement of the beam in lead VL (fig. 2B).

This method permits a rough delimitation of those regions of the heart which when made negative at their surface result in a downward movement of the beam in this lead (the proximal zone), and those regions which when made negative at their surface result in an upward movement of the beam (the distal zone). More exact delimitation of the location and extent of the various zones is described below.

Received for publication April 16, 1948.

¹ Aided by grants from the Fluid Research Fund, Yale University School of Medicine, and the U. S. Public Health Service.

² Senior Research Fellow, U. S. Public Health Service.

Forced Epicardial Extrasystoles

Stimulation of many points on the anterior and posterior surfaces of both ventricles yielded three different types of extrasystoles.

Type A (fig. 3A). The ventricular complex exhibits a QS deflection and an upright T-wave. This type of extrasystole could be obtained only by stimulation of points on the upper two-thirds of the anterior right and left ventricle, and from the extreme basal portion of both ventricles posteriorly.

Type B (fig. 3C). The ventricular complex exhibits a large R-wave followed by

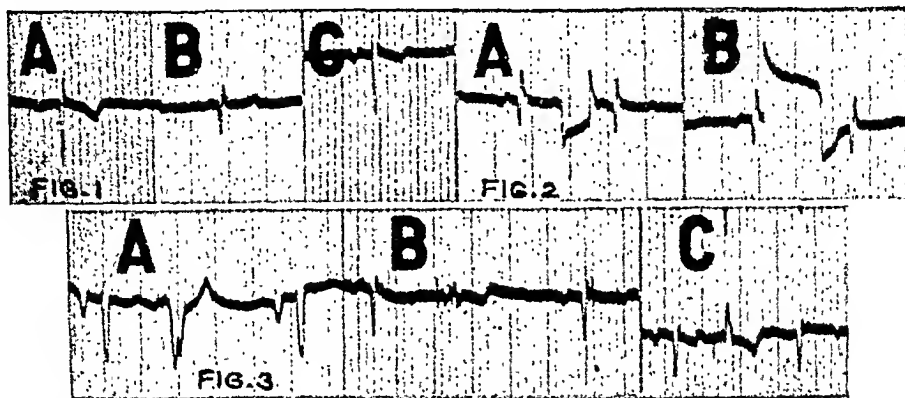


Fig. 1. A, B, AND C ARE VARIOUS NORMAL PATTERNS seen in dogs under Dial anaesthesia before the chest is opened. There are variations in the QRS complex and in the direction of the T-wave.

Fig. 2. DOG, MALE, 6.5 KGM., 10/23/47. One electrode on various points of the upper two-thirds anterior surface of the heart and another electrode on various points lower two-thirds posterior surface of the heart. A, impressed negativity at electrode on anterior surface. The beam moves downward. B, impressed negativity at electrode on posterior surface. The beam moves upward.

Fig. 3. PATTERNS OF EXTRASYSTOLES elicited by stimulation of the epicardial surface of the various segments of the heart. Dog, female, 6.0 kgm., 10/28/47. A, extrasystoles elicited from the upper two thirds of the anterior surface of the right and left ventricles, and from the extreme basal portion of both ventricles posteriorly. Initial movement of the beam is downward. B, extrasystoles elicited from the anterior surface of both ventricles just above the apex, and from the posterior surface of both ventricles near the base. Small initial upward deflection followed by downward deflection. C, extrasystoles elicited from the right and left apex, anterior and posterior, and from the lower two thirds of the posterior surface of both right and left ventricles. Initial upward movement of the beam.

an inverted T-wave. This type of extrasystole could be obtained only by stimulation of points on the anterior and posterior right and left apex, and the lower two-thirds of the posterior surface of both right and left ventricles.

Type C (fig. 3B). The ventricular complex exhibits an rS or qRs complex, usually followed by an upright T-wave. This type of extrasystole could be obtained only by stimulation of points in a narrow segment across the anterior surface of the heart just above the apex and from a narrow segment across the posterior surface of the heart near the base.

The heart can, therefore, be divided into three distinct zones for lead VL (fig. 4). That portion of the heart from which extrasystoles of the QS pattern are obtained is designated as the proximal zone of lead VL; that portion of the heart from which extrasystoles of the R pattern are obtained is designated as the distal zone; that por-

tion from which extrasystoles of the rS or qRs pattern are obtained is designated as the intermediate zone. When the first portion of the heart to become depolarized lies in the proximal zone, the initial movement of the beam is downward. Conversely, when depolarization first occurs in a portion of the distal zone, the beam first moves upward.

Effect upon ST-segment of Surface Injury from Application of M/5 KCl or Superficial Cautery

ST-segment elevation. This is obtained following production of epicardial injury in the upper two-thirds of the anterior surface of the right and left ventricles (the proximal zone, fig. 5A).

ST-segment depression. This is only obtained following injury to the right or left apex or the lower two-thirds of the posterior surface of the heart (the distal zone, fig. 5C).

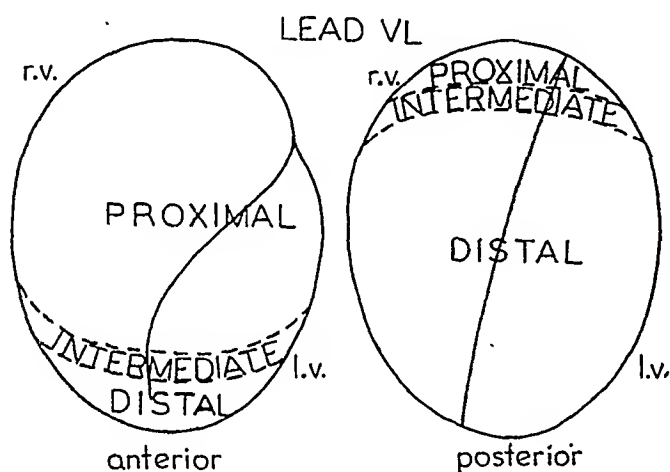


Fig. 4. SCHEMATIC DRAWING of the anterior and posterior surfaces of the dog heart outlining the location and extent of the proximal, intermediate and distal zones of lead VL.

No ST-segment deviation. This usually occurs when injury is produced just above the apex anteriorly or near the extreme base posteriorly (the intermediate zone, fig. 5B).

Immediate Effects of Coronary Ligation on ST-segment

The following observations were made after coronary ligation:

Occlusion right coronary artery. This usually results in ischemia of the upper half of the anterolateral wall of the right ventricle. The ST-segment becomes elevated (fig. 6A).

Occlusion posterior descending branch right coronary artery. This is usually followed by ischemia of a portion of the posterior right ventricle. The ST-segment may be slightly elevated (fig. 6B).

Occlusion anterior descending branch left coronary artery. This is usually followed by ischemia of the anterior septal area. The ST-segment is elevated (fig. 6C).

Occlusion lateral descending branch left coronary artery. This usually produces ischemia of the anterolateral wall of the left ventricle. The ST-segment usually shows little change (fig. 6D).

Occlusion posterior descending branch left coronary artery. This usually produces ischemia of the posterior left ventricle and the posterior left apex. The ST-segment is depressed (fig. 6E).

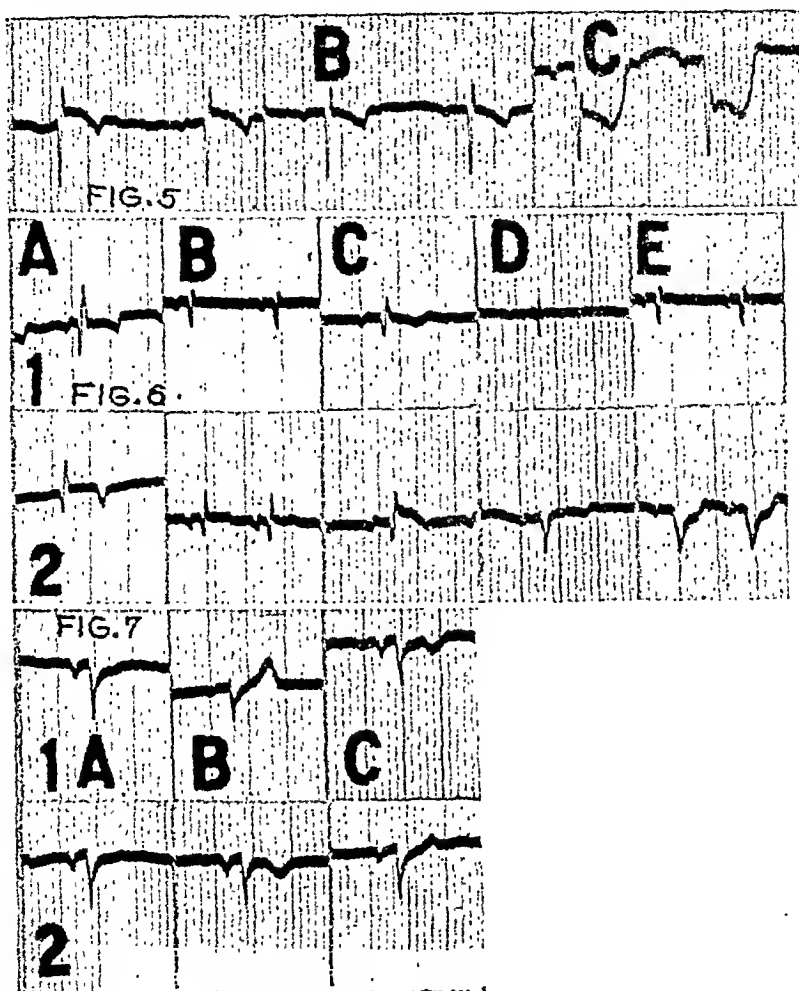


Fig. 5. ST-SEGMENT DEVIATIONS following application of M/5 KCl to various parts of the heart surface. Dog, male, 9.0 kgm., 7/31/47. A, proximal zone, ST-segment elevated. B, intermediate zone, ST-segment isopotential. C, distal zone, ST-segment depressed.

Fig. 6. ST-SEGMENT DEVIATIONS following occlusion of different coronary vessels. 1, control; 2, following ligation. A, ligation right coronary artery. Dog, male, 7.5 kgm., 1/6/48. ST-segment is slightly elevated. B, ligation circumflex branch right coronary artery. Dog, female, 4.1 kgm., 2/17/48. ST-segment isopotential. C, ligation anterior descending branch left coronary artery. Dog, male, 11.4 kgm., 8/15/47. ST-segment elevated. D, ligation lateral branch left coronary artery. Dog, female, 8.4 kgm., 12/11/47. ST-segment isopotential. E, ligation posterior descending branch left coronary artery. Dog, female, 4.1 kgm., 2/17/48. ST-segment depressed.

Fig. 7. EFFECT OF HEATING AND COOLING proximal and distal zones on the direction of the T-wave. Dog, male, 7.4 kgm., 9/4/47. 1, chamber on proximal zone; 2, chamber on distal zone. A, control. B, water at 50°C. circulating through chamber. Heating proximal zone, T-wave more upright; heating distal zone, T-wave inverted. C, water at 20°C. circulating through chamber. Cooling proximal zone inverts T-wave. Cooling distal zone makes T more upright.

When injury is limited to the proximal zone of lead VL the ST-segment is found to be elevated. Injury limited to the distal zone results in depression of the ST-seg-

ment. When injury extends into both zones there may be no deviation of the ST-segment or the direction of deviation may vary, depending upon the relative extent of injury in each zone.

Effect upon T-wave of Heat and Cold on Epicardial Surface

When repolarization of the proximal zone is accelerated by heating the epicardial surface of the upper two-thirds of the anterior right and left ventricles, the T-wave becomes more positive (fig. 7). When repolarization of this zone is slowed by cooling it, the T-wave becomes more negative. When repolarization of the distal zone is accelerated by heating the epicardial surface of the apex or the lower two-thirds of the posterior right and left ventricles, the T-wave becomes more negative. Delaying the repolarization of this zone by cooling it results in increased positivity of T.

DISCUSSION

The direction of beam movements in lead VL is found to vary with the spatial relation of the region of the heart being depolarized to the position of the exploring electrode on the left forelimb. When depolarization occurs in any portion of the proximal zone (the upper two-thirds of the anterior surface of the right and left ventricles and the extreme basal portion of the posterior surface of the heart), the beam moves downward. When depolarization occurs in any portion of the distal zone (the apex and the lower two-thirds of the posterior surface of the heart), the beam moves upward. Depolarization occurring in the intermediate zone which separates the proximal and distal zones may result in no movement of the beam.

The factors which determine the position of the beam at any given moment during the excitation and recovery process have already been discussed in detail in the preceding communication dealing with the nature of lead VR (2).

Knowledge of the areas of the heart which lie in the proximal, intermediate and distal zones of lead VL permits the following analysis of the deflections which occur in the normal ventricular complex in this lead. Lead VL usually exhibits a Qr complex followed by an inverted or upright T-wave (fig. 1). The initial downward deflection indicates that excitation first occurs in a portion of the proximal zone (the upper two-thirds of the anterior surface of the heart). The reversal of the direction of beam movement producing the upstroke of the Q-wave to the peak of the R-wave indicates spread of the excitatory process to involve regions lying in the distal zone (the apex and the major portion of the posterior surface of the heart). The downstroke of the R-wave indicates terminal excitation again of some portion of the proximal zone. More specific localization of the spread of the excitatory process will be presented in a subsequent communication from analysis of leads VR, VL and VF recorded simultaneously.

The factors which may be responsible for the isopotential ST-segment cannot be determined from an analysis of lead VL alone (see lead VR (2) for discussion of isopotential ST-segments). An upright T-wave in this lead indicates that repolarization in the distal zone is proceeding more slowly than in the proximal zone. Conversely, an inverted T-wave indicates that repolarization of the proximal zone is proceeding more slowly than that of the distal zone.

The experiments herein described confirm the conclusions reached in the preceding communication that variations in cavity potential cannot be the basis for the origin of the 'unipolar' extremity lead electrocardiogram. Forced extrasystoles from the upper two-thirds of the anterior surface of the left ventricle exhibit a QS deflection, while those elicited from the posterior surface of this ventricle as well as from the apex show only an R-complex. Since all these points on the anterior and posterior epicardial surface are in the same relation to the cavity of the left ventricle, the extrasystoles should all exhibit an initial R-wave in a lead which is considered to reflect the variations in potential of the left cavity. In each case the cavity would remain electropositive until the excitatory process had spread through the thickness of the left ventricle and had reached the endocardial surface. Forced extrasystoles from the upper two-thirds of the anterior right ventricle also exhibit a QS deflection, whereas if variation in potential of the left ventricular cavity determined the nature of lead VL, there should be an initial R-wave in the extrasystole since the cavity would remain electropositive until the excitatory process initiated on the surface of the right ventricle had finally spread to the endocardial surface of the left ventricle. Injury to the anterior surface of the heart invariably produces an elevation of the ST-segment in lead VL. Since the cavity of the left ventricle is 'facing-away' from the injured surface and would therefore be in the positive field, an ST-segment depression would be expected.

The conclusions here reached about the origin of the T-wave are completely at variance with explanations current in the literature (3, 4) which hold that the configuration of the T-wave may be caused by differences in rates of repolarization of the endocardial and epicardial lamellae of the heart. According to this view, an upright T-wave in a 'unipolar' lead which 'faces' the cavity of the heart results when the endocardium repolarizes more rapidly than the epicardium; conversely, an inverted T-wave results when the epicardium repolarizes more rapidly than the endocardium. This concept is rendered untenable by the following observations: *a*) accelerating the repolarization at the epicardial surface of the anterior right and left ventricle by heat results in a more positive T-wave rather than a more negative one; *b*) slowing repolarization of the epicardial surface of the anterior right and left ventricles by cooling it results in a more negative T-wave rather than a more positive one; *c*) accelerating the repolarization at the epicardial surface of the posterior left ventricle by heat results in increased negativity of the T-wave while similar treatment of the anterior surface of the left ventricle results in increased positivity of the T-wave. In each instance repolarization of the epicardium is accelerated although the alteration in T-wave is in the opposite direction.

SUMMARY

1. Lead VL is inscribed as a result of interference between electrical events due to depolarization occurring in regions of the heart 'facing' the exploring electrode (the proximal zone), which cause downward movement of the beam, and electrical events occurring in regions 'facing away' from the exploring electrode (the distal zone), which cause upward movement of the beam.
2. For lead VL the proximal zone consists of the upper two-thirds of the anterior

right and left ventricles and the extreme basal portion of the posterior surface of both ventricles. The distal zone consists of the right and left apex and the lower two-thirds of the posterior surface of the heart. There is an intermediate zone separating the proximal and distal zones which consists of a narrow segment across the anterior surface of the heart just above the apex, and a narrow segment across the posterior surface of the heart near the base.

3. The spread of excitation and recovery in the various parts of the dog heart has been roughly determined from an analysis of the position of the beam in this lead at successive moments in the ventricular complex.

4. Injury to the heart can be localized by a study of ST-segment deviation. Elevation of the ST-segment indicates existence of injury in the proximal zone, while depression of the ST-segment indicates injury in the distal zone.

5. Localization of site of origin of ventricular extrasystoles may be determined from their configuration. Those that arise in the proximal zone exhibit a QS complex; those that arise in the distal zone exhibit an R complex; while those that arise in the intermediate zone exhibit an rS or qRs complex.

6. The order of repolarization in the heart can be determined from the configuration of the T-wave. An upright T-wave indicates that the proximal zone repolarizes more rapidly than the distal zone. An inverted T-wave indicates that the proximal zone repolarizes more slowly than the distal zone.

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NATURE OF 'UNIPOLAR' EXTREMITY LEADS IN THE DOG¹ LEAD VF

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IN THE dog the 'unipolar' extremity lead VF normally exhibits one of several patterns (fig. 1). These correspond closely with the patterns usually seen in man. It has already been demonstrated that the 'unipolar' extremity leads VR and VL (1, 2), as well as 'unipolar' precordial leads (3), develop as a result of interference between electrical events due to depolarization occurring in specific proximal, intermediate and distal regions of the heart, and that the location and extent of these regions vary with the position of the exploring electrode.

In order to determine the derivation of lead VF it is, therefore, only necessary to delimit the areas of the heart which lie in the proximal, intermediate and distal zones of this particular lead. To accomplish this, methods which have been described previously were employed (1).

Lead VF was recorded by pairing an exploring electrode inserted subcutaneously in the left hindlimb with a central terminal electrode constructed as described previously (1).

RESULTS

Impressed Potentials

When one electrode was placed at any point on the surface of the left apex or on the posterior left ventricle and the other electrode at any point on the surface of the right ventricle, negativity of the electrode on the left ventricle and positivity of the electrode on the right ventricle caused a downward movement of the beam in lead VF. Reversal of polarity so that the electrode on the left ventricle was positive resulted in an upward movement of the beam (fig. 2). This method permits a rough delimitation of those regions of the heart surface which, when made electronegative, result in downward movement of the beam, and those regions of the heart surface which, when made electronegative, result in upward movement of the beam.

Forced Epicardial Extrasystoles

Stimulation of selected points on the epicardial surface of both ventricles, anterior and posterior, yielded three different types of extrasystoles.

Type A (fig. 3A). The ventricular complex exhibits a QS complex and an upright T-wave. This type of extrasystole can be obtained only by stimulation of

Received for publication April 16, 1948.

¹ Aided by grants from the Fluid Research Funds, Yale University School of Medicine, and the U. S. Public Health Service.

² Senior Research Fellow, U. S. Public Health Service.

points on the anterior and posterior left apex, the lower third of the anterior left ventricle and the posterior left ventricle.

Type B (fig. 3C). The ventricular complex exhibits a large R-wave followed by an inverted T-wave. This type of extrasystole could be obtained only by stimulation of points on the anterior and posterior right ventricle (except the right apex) and the upper third of the anterior left ventricle.

Type C (fig. 3B). The ventricular complex exhibits an rS or qRs complex usually followed by an upright T-wave. This type of extrasystole could be obtained only by stimulation of points on the right apex, the mid-third of the anterior left ventricle and the posterior septal area.

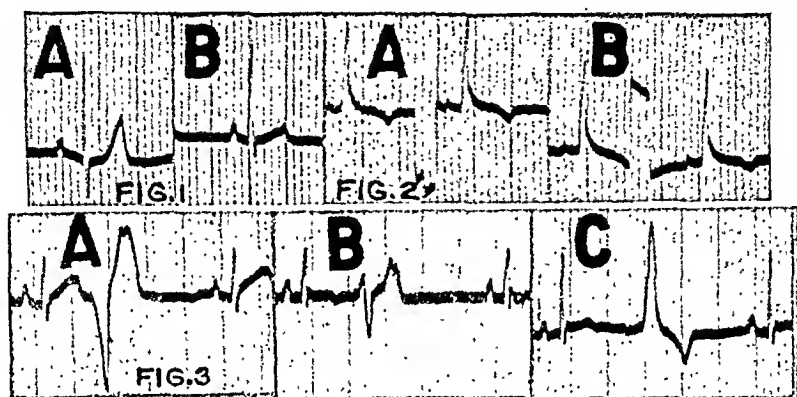


Fig. 1. A and B ARE THE COMMONEST PATTERNS seen in lead VF in the anesthetized dog. There are slight variations in the QRS complex. The T-wave is directed upright.

Fig. 2. DOG, 4.6 KGM., MALE, 10/9/47. One electrode on posterior left apex and another electrode on surface right lateral ventricle. A, impressed negativity at electrode on posterior left apex. The beam moves downward. B, impressed negativity at electrode on right ventricle. The beam moves upward.

Fig. 3. PATTERNS OF EXTRASYSTOLES elicited by stimulation of the epicardial surface of the various segments of the heart. Dog, 6.5 kgm., male, 10/23/47. A, extrasystoles elicited from the left apex, anterior and posterior, and from the posterior left ventricle. Initial movement of the beam is downward. B, extrasystoles elicited from the right apex, the mid-third of the anterior left ventricle, and from the posterior septal area. Small initial upward deflection followed by downward deflection. C, extrasystoles elicited from the right ventricle, anterior and posterior, and from the basal portion of the left anterior ventricle. Initial movement of the beam is upward.

That portion of the heart from which extrasystoles of the QS pattern are obtained is designated as the proximal zone of lead VF; that portion of the heart from which extrasystoles of the R pattern are obtained is designated as the distal zone; and that portion of the heart from which extrasystoles of the rS or qRs pattern are obtained is designated as the intermediate zone (fig. 4).

Effect upon ST-segment of Surface Injury from Application of M/5 KCl or Superficial Cautery

ST-segment elevation. This follows production of injury of the epicardial surface of the left apex, of the lower third of the anterior left ventricle and of the posterior left ventricle (the proximal zone, fig. 5A).

ST-segment depression. This follows production of injury of the epicardial sur-

face of the anterior and posterior right ventricle and the upper third of the anterior left ventricle (the distal zone, fig. 5C).

No effect upon the ST-segment. This usually follows production of injury of the epicardial surface of the right apex, the mid-third of the anterior left ventricle and the posterior septal area (the intermediate zone, fig. 5B).

Immediate Effects of Coronary Ligation on ST-segment

Occlusion right coronary artery. Ischemia of the anterolateral wall of the right ventricle usually occurs. The ST-segment is found to be depressed (fig. 6A).

Occlusion posterior descending branch right coronary artery. Ischemia of a portion of the posterior right ventricle usually occurs. The ST-segment is found to be depressed (fig. 6B).

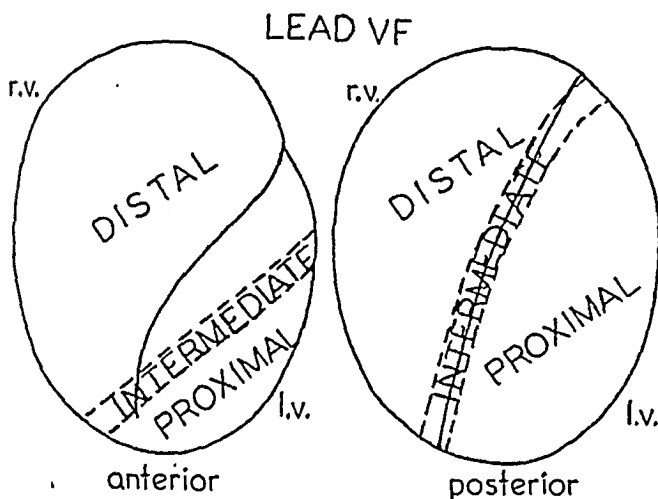


Fig. 4. SCHEMATIC DRAWING of the anterior and posterior surfaces of the dog heart outlining the location and extent of the proximal, intermediate and distal zones of lead VF.

Occlusion anterior descending branch left coronary artery. Ischemia of the anterior septal region occurs. The ST-segment is usually slightly depressed (fig. 6C).

Occlusion lateral descending branch left coronary artery. Ischemia of the anterolateral wall of the left ventricle occurs. The ST-segment is found to be elevated (fig. 6D).

Occlusion posterior descending branch left coronary artery. Ischemia of a portion of the posterior left ventricle occurs. The ST-segment is found to be elevated (fig. 6E).

Thus when injury is limited to regions of the heart which are in the proximal zone of lead VF, the ST-segment is found to be elevated; when injury is limited to regions lying in the distal zone, the ST-segment becomes depressed; when injury extends into portions of both zones, the direction of ST-segment deviation will vary, depending upon the relative involvement of each zone.

Effect upon T-wave of Heat and Cold on Epicardial Surface

When repolarization of the proximal zone is accelerated by heating the epicardial surface of the left apex and the posterior left ventricle, the T-wave becomes more positive (fig. 7). When repolarization of this zone is delayed by cooling it, the T-wave

becomes more negative. When repolarization of the distal zone is accelerated by heating the epicardial surface of the right ventricle, the T-wave becomes more nega-

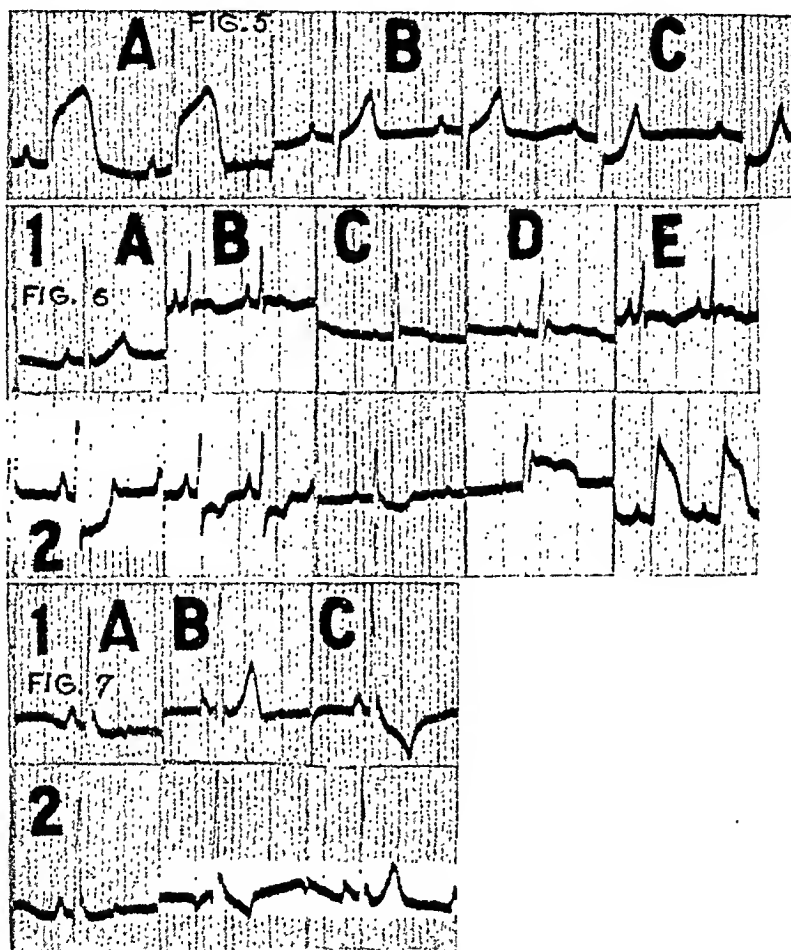


Fig. 5. ST-SEGMENT DEVIATIONS following application of M/5 KCl to various parts of the heart surface. Dog, 9.0 kgm., male, 7/31/47. A, proximal zone, ST-segment elevated. B, intermediate zone, ST-segment isopotential. C, distal zone, ST-segment depressed.

Fig. 6. ST-SEGMENT DEVIATIONS following occlusion of various vessels. 1, control; 2, following ligation. A, ligation right coronary artery. Dog, 7.5 kgm., male, 1/6/48. ST-segment is depressed. B, ligation circumflex branch right coronary artery. Dog, 4.1 kgm., female, 2/17/48. ST-segment is depressed. C, ligation anterior descending branch left coronary artery. Dog, 6.6 kgm., male, 9/2/47. ST-segment slightly depressed. D, ligation lateral branch left coronary artery. Dog, 5.6 kgm., female, 8/21/47. ST-segment elevated. E, ligation posterior descending branch left coronary artery. Dog, 4.1 kgm., female, 2/17/48. ST-segment elevated.

Fig. 7. EFFECT OF HEATING AND COOLING proximal and distal zones on the direction of the T-wave. Dog, 6.6 kgm., female, 10/2/47. 1, chamber on proximal zone; 2, chamber on distal zone. A, control. B, water at 50°C. circulating through chamber. Heating proximal zone, T-wave more upright. Heating distal zone, T-wave inverted. C, water at 20°C. circulating through chamber. Cooling proximal zone inverts T-wave. Cooling distal zone makes T-wave more upright.

tive. Conversely, delaying the repolarization of this zone by cooling it results in increased positivity of the T-wave.

DISCUSSION

The initial direction of beam movement in lead VF, just as it has been found for leads VR and VL, varies with the spatial relation of the regions of the heart being depolarized or repolarized to the position of the exploring electrode situated on the left hindlimb. When depolarization occurs in the areas of the left apex, the lower third of the anterior left ventricle and the posterior left ventricle (the proximal zone), the beam moves downward. When depolarization occurs in the areas of the anterior and posterior right ventricle and the upper third of the anterior left ventricle (the distal zone), the beam moves upward. Depolarization of the right apex, the mid-third of the anterior left ventricle and the posterior septal area (the intermediate zone) may result in no movement of the beam.

This knowledge of the areas of the heart which lie in each zone of lead VF permits an analysis of the deflections which normally occur in this lead. The ventricular complex seen in this lead usually consists of a large R-wave followed by a small S-wave, an isoelectric ST-segment and an upright T-wave. The initial upward deflection, therefore, indicates that initial excitation is occurring in portions of the heart which lie in the distal zone of this lead, namely, the right ventricle and the upper third of the anterior left ventricle. The downstroke of R is inscribed when the excitation process has spread preponderately to involve portions of the heart which lie in the proximal zone of this lead, namely, the left apex and the posterior left ventricle. The terminal reversal of the direction of the beam to inscribe the upstroke of S indicates late spread of the excitatory process to some portion of the heart which lies in the distal zone. More specific localization of the spread of excitation will be discussed in a subsequent communication dealing with analysis of several simultaneously recorded 'unipolar' extremity leads. The upright T-wave indicates that repolarization begins first in that portion of the heart which lies in the proximal zone of this lead, namely, the left apex and the posterior left ventricle.

The results of these experiments do not throw any further light on the validity of the 'cavity potential' theory. Since the exploring electrode in lead VF is considered to 'face' the epicardial surface of the left ventricle, the experimental findings might be as well explained by the 'cavity potential' theory as by the 'zonal interference' theory. However, the 'cavity potential' theory of the derivation of the ventricular complex has already been found to be invalid in the studies dealing with the nature of the precordial electrocardiogram (3) and the 'unipolar' extremity leads VR and VL (1, 2), and therefore it seems logical to explain the nature of lead VF also on the theory of 'zonal interference.'

SUMMARY

1. Lead VF is inscribed as a result of interference between electrical events due to depolarization occurring in regions of the heart 'facing' the exploring electrode (the proximal zone), which cause downward movement of the beam, and electrical events occurring in regions of the heart 'facing away' from the exploring electrode (the distal zone), which cause upward movement of the beam.

2. For lead VF the proximal zone consists of the left apex, the lower third of the

anterior left ventricle and the posterior left-ventricle. The distal zone consists of the anterior and posterior right ventricle and the upper third of the anterior left ventricle. There is an intermediate zone which lies between the proximal and distal zones, which consists of the right apex, mid-third of the anterior septum and anterior left ventricle, and the posterior septal area.

3. The spread of excitation and recovery in the various parts of the dog heart has been roughly determined by analyzing the position of the beam at successive moments in the ventricular complex.

4. Injury to the heart can be localized by study of the ST-segment deviation. Elevation of the ST-segment indicates injury in the proximal zone, while depression of the ST-segment indicates injury in the distal zone.

5. Localization of the site of origin of ventricular extrasystoles may be determined from their configuration. Those which arise in the proximal zone exhibit a QS complex; those which arise in the intermediate zone exhibit an rS or qRs complex; those which arise in the distal zone exhibit an R complex.

6. The order of repolarization in the heart can be determined from the configuration of the T-wave. An upright T-wave results when the proximal zone repolarizes more rapidly than the distal zone. An inverted T-wave results when the proximal zone repolarizes more slowly than the distal zone.

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FACTORS INVOLVED IN THE PRODUCTION OF PAROXYSMAL VENTRICULAR TACHYCARDIA INDUCED BY EPINEPHRINE

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AFTER the usual primary rise in blood pressure and the reflex bradycardia which follow the injection of epinephrine into unanesthetized animals, a marked secondary increase in pressure followed by a paroxysm of ventricular tachycardia can be seen (1). We have already shown that the degree and duration of the secondary pressure rise and tachycardia depend primarily upon the amount of epinephrine injected (2). The sudden onset, within a beat or two, of these phenomena suggested that a neurogenic mechanism might be responsible for their production. This concept is supported by our findings that the ventricular tachycardia is inhibited by atropine (1), pentobarbital anesthesia, dibenamine (3) and to some extent by morphine (2). However, the mode of production of the ventricular tachycardia was not well understood. We, therefore, undertook electrocardiographic and blood pressure studies on several types of preparations in order to analyze the factors responsible for such aberrant cardiac action.

METHODS

Nine dogs weighing from 10 to 20 kgm. were anesthetized with morphine sulfate 1 mgm/kgm. and chloralose 100 mgm/kgm. Blood pressures were measured from the femoral artery with the Hamilton manometer and the electrocardiogram (lead 2) recorded with a direct-writing electrocardiograph (Viso-cardiette). Electrocardiographic records were obtained before, during and after the injection of epinephrine. Blood pressures were recorded continuously. This permitted a close correlation between the two sets of records.

RESULTS

1. *Effects of Anesthesia on Paroxysmal Ventricular Tachycardia.* It was necessary to use an anesthetic agent which would not prevent the paroxysmal phenomena. This was accomplished by the use of chloralose which has been shown to produce general anesthesia without inhibiting the carotid sinus reflexes (4). Morphine was also given in order to obtain a more complete relaxation and to enhance vagal tone. We have shown (2) that morphine tends to partially inhibit the paroxysmal tachycardia. After a control electrocardiogram 1 mgm. of epinephrine was injected in-

Received for publication April 13, 1948.

¹ Graduate Fellow of the Belgian American Educational Foundation.

This department is supported in part by the Michael Reese Research Foundation.

² Aided by the A. D. Nast Fund for Cardiovascular Research.

travenously to determine whether paroxysmal ventricular tachycardia could be produced in each anesthetized animal. In several animals which showed a typical paroxysm with epinephrine in the unanesthetized state, the paroxysms could not be elicited after chloralose-morphine anesthesia. These animals were not used in the present study. Sinus bradycardia and varying degrees of A-V block were, however, present in all animals after the injection of epinephrine. The blood pressure and electrocardiographic findings were similar to those in the unanesthetized animal with the exception that the rate of the paroxysmal ventricular tachycardia did not exceed 220/minute while without any anesthesia rates up to 400 beats/minute have been observed.

2. *Effect of Vagotomy.* Both vagi were isolated after the control epinephrine injection in the anesthetized animals. Epinephrine was then again injected and as soon as ventricular tachycardia appeared both vagi were cut, either simultaneously or successively. In 3 animals in which only one vagus nerve was cut during the paroxysmal ventricular tachycardia, the paroxysm was unaffected. In these animals with unilateral vagotomy, paroxysmal ventricular tachycardia could be obtained with succeeding epinephrine injections. During a subsequent tachycardia induced by epinephrine the remaining vagus was sectioned. In each instance the tachycardia stopped within a few beats. (Subsequent injections of epinephrine did not elicit the ventricular tachycardia.)

In 3 animals in which both vagi were cut simultaneously a few seconds after the onset of the epinephrine induced tachycardia, the paroxysmal ventricular tachycardia stopped abruptly within 4 to 8 beats. It was replaced by sinus tachycardia or auricular fibrillation with a rate more rapid than that of the preceding ventricular tachycardia. The injection of epinephrine into the bilaterally vagotomized animal now produced only an acceleration of the sinus rate, but never a ventricular tachycardia. In several instances, however, single ectopic beats were seen.

3. *Effect of Vagal Stimulation or Acetylcholine Injection.* In 12 experiments epinephrine injection was followed by electrical stimulation of the peripheral end of both cut vagi. The effect of this peripheral vagal stimulation depended on the time which had elapsed since the injection of epinephrine. Stimulation during the first 15 to 40 seconds produced either cardiac standstill as is ordinarily seen with vagal stimulation or 1 to 3 ventricular escapes followed by cardiac standstill or a slow idioventricular rhythm. Not infrequently auricular fibrillation was manifest at the end of vagal stimulation. When 40 to 60 seconds elapsed after the injection of epinephrine, paroxysmal ventricular tachycardia sometimes appeared during the vagal stimulation. The rate of this tachycardia was equal to that of the paroxysmal ventricular tachycardia observed before vagotomy. However, it was slower than the ventricular rate observed during the sinus tachycardia or auricular fibrillation induced by section of the vagi. The first ectopic ventricular beat was preceded by an R-R interval longer than that of the preceding beats. The ventricular tachycardia lasted either throughout the period of vagus stimulation or stopped abruptly to give way to cardiac standstill or to a slow ventricular pacemaker. When more than 3 minutes elapsed between the injection of epinephrine and vagal stimulation, ventricular tachycardia could not be elicited by the vagal stimulation. The sensitive period in which vagal stimulation

elicited ventricular tachycardia corresponded to the usual time of occurrence of ventricular tachycardia in the intact animal.

Ventricular tachycardia could also be produced by the intravenous injection of 1 to 10 mgm. of acetylcholine, injected at similar periods after the epinephrine injection. Ventricular tachycardia was obtained in 8 trials when the acetylcholine was injected about 15 to 100 seconds after the injection of epinephrine. This corresponded with the time when ventricular tachycardia occurred after epinephrine in the animal with intact vagi. This tachycardia was usually preceded by sinus bradycardia and A-V block but sometimes appeared abruptly without such a transition.

4. *Effect of Maintaining the Blood Pressure at a Constant Level.* In 3 anesthetized dogs with vagi intact the abdominal aorta was exposed through a midline abdominal incision. A 'T' cannula was introduced into the aorta between the renal and the iliac arteries, and connected to a pressure equalizer. The equalizer was designed so that as the blood pressure in the animal tended to rise after the injection of epinephrine, blood would flow rapidly into the reservoir, resulting in no appreciable change in arterial pressure. As the pressure tended to fall in the animal, blood flowed from the equalizer back into the animal, with no change in pressure. Our equalizing system consisted of an air reservoir connected to an aspirator bottle and to a mercury manometer. The outlet at the bottom of the aspirator bottle was connected by means of a short wide rubber tubing to the cannula in the aorta. In these experiments blood pressure was recorded from the aorta instead of the femoral artery. With the equalizer connected and the pressure in the bottle equal to that in the aorta, 1 mgm. of epinephrine was injected intravenously. The maintenance of constant pressure caused a loss of 300-500 cc. of blood into the aspirator bottle. After the epinephrine effect wore off the same or a slightly larger amount of fluid returned to the animal.

The injection of epinephrine under these conditions caused a slight acceleration of the sinus rate rather than the usual slowing. No ectopic beats or rhythms appeared.

5. *Effect of a Sudden Increase in Blood Pressure by Abrupt Infusion of Large Amounts of Fluid.* In 3 experiments at times when no epinephrine had been injected, the aortic pressure was raised suddenly for a few seconds by quickly increasing the pressure in the equalizer and allowing 300-500 cc. of fluid to flow into the animal. The pressure was increased instantly, but could be kept up only during the few seconds of rapid infusion. Immediately on cessation of the rapid inflow the blood pressure quickly returned to the pre-infusion level. The sudden rise in pressure caused only a momentary sinus bradycardia and the rate accelerated as soon as the pressure began to fall. No ectopic beats or rhythms developed.

Similarly produced rises in blood pressure after epinephrine was injected led to a sinus bradycardia followed by a nodal rhythm and, after a few seconds, by a paroxysm of ventricular tachycardia.

In 1 animal in which we were unable to obtain ventricular tachycardia with epinephrine alone it was found that $2\frac{1}{2}$ minutes after the injection of epinephrine a sudden rise in blood pressure produced by the rapid infusion of 300 cc. of blood precipitated a paroxysm of ventricular tachycardia within one second.

DISCUSSION

Two factors, an increased irritability of the idioventricular pacemakers and a depression of the sinus and A-V nodal pacemakers, appear necessary to explain the occurrence of paroxysmal ventricular tachycardia in our type of experiments. This is in accord with the analysis of Hoff and Nahum (5) and with the general concept first enunciated by Rothberger and Winterberg (6) over 30 years ago.

The idioventricular pacemakers are normally suppressed by the more rapidly discharging higher pacemakers residing in the S-A and A-V nodes. Endogenous or exogenous stimulants such as epinephrine tend to excite the entire system of rhythmically discharging pacemakers and permit, under favorable circumstances, the appearance of active ectopic rhythms. Another factor which may have played a rôle in our experiments may have arisen from the direct stretching of the myocardium which is caused by the marked and abrupt blood pressure rise.

The inhibition of the higher pacemakers is dependent upon the vagus or upon the introduction into the body of endogenous or exogenous sources of acetylcholine. The vagus may be stimulated to increased activity through reflexes arising in pressor receptors. The increase in pressure attendant upon the injection of epinephrine or large amounts of fluid acts reflexly via the carotid sinus to produce vagal inhibition of the heart which is observed in the slowing of the sinus rate and the tendency for A-V block. The injection of acetylcholine produces similar effects through its action on the vagus.

Our studies, like those of Hoff and Nahum, indicate that the two factors under discussion must act simultaneously in order to precipitate a paroxysm of ventricular tachycardia. Epinephrine alone does not produce this tachycardia in the vagotomized or atropinized animal because the higher pacemakers are not depressed. The prevention of the epinephrine pressure rise with the equalizer eliminates the carotid sinus reflex via the vagus and is thus comparable in its effects to that of atropine. The prevention of ventricular tachycardia by means of a pressure stabilizer has also been demonstrated recently in the cyclopropane anesthetized dog by Moe *et al.* (7). Vagal stimulation alone fails to induce ventricular tachycardia because those ventricular pacemakers which are responsible for the ventricular tachycardia are not stimulated. The exhibition of epinephrine followed by vagal stimulation either reflexly in the intact animal or directly in the vagotomized animal provide the prerequisites necessary for the production of paroxysmal ventricular tachycardia. Our experiments show that the balance between depression of the higher and excitation of the lower pacemakers is favorable for the production of ventricular tachycardia only during a short period of time after the administration of epinephrine.

Hoff and Nahum (5) also showed that the ectopic pacemaker must have a faster rate than the normal pacemaker in order to take over the control of the heart. Vagotomy during the ventricular tachycardia shows how this ectopic pacemaker can be immediately suppressed by a faster sinus rate. While the rate of the ventricular tachycardia obtained by vagal stimulation in the vagotomized animal was slower than the preceding sinus rate this apparent contradiction is easily explained by the fact that the higher pacemakers are immediately suppressed by vagal stimulation. Since

the first beat in such a tachycardia is always preceded by an R-R interval longer than that of the preceding beats, this beat must be considered as a ventricular escape caused by the sudden depression of A-V conduction and not as a vagal stimulation of the ventricle. If at this time the ventricle is sufficiently excited by epinephrine this ventricular escape leads into a ventricular tachycardia.

SUMMARY AND CONCLUSION

Various factors involved in the paroxysmal ventricular tachycardia induced by epinephrine were studied in the dog. Bilateral vagotomy during a paroxysm of ventricular tachycardia stopped this tachycardia within a few beats in all animals. Neither epinephrine nor vagal stimulation alone are capable of inducing paroxysmal ventricular tachycardia in the vagotomized animal. Peripheral vagal stimulation or acetylcholine may produce ventricular tachycardia after the injection of epinephrine. Maintenance of constant pressure after the injection of epinephrine by the use of a pressure equalizer prevents the occurrence of ectopic rhythms. A sudden rise in pressure during this period precipitates ventricular tachycardia. A rise in pressure without the injection of epinephrine does not produce ventricular tachycardia.

It is concluded that two factors are involved in the epinephrine induced ventricular tachycardia: *a*) hyperexcitability of the idioventricular pacemakers due to the presence of epinephrine—stretching of the myocardium due to the sudden increase in pressure may contribute to this effect; *b*) inhibition of the higher pacemakers through vagal stimulation.

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PRODUCTION OF EXPERIMENTAL HEART FAILURE IN DOGS WITH INTACT CIRCULATION¹

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THE manifestations and mechanism of congestive heart failure have held the interest of many investigators. Despite numerous studies, the facts immediately concerned in the precipitation of myocardial insufficiency are not clear. In the heart-lung preparation, with its controlled circulation and lack of nervous influences these factors are better known. In the heart-lung preparation substances such as chloroform (1) or chloral hydrate (2) frequently provoke acute heart failure as shown by cardiac dilatation and pulmonary edema. Interruption of the coronary circulation by ligation or by drugs (3, 4) often produces similar results if ventricular fibrillation can be avoided. It is evident that in the heart-lung preparation, severe myocardial damage may lead to failure of the heart.

However, these data cannot be applied to studies of heart failure without further investigation of the innervated heart with intact circulation. Using the intact animals, congestive failure is difficult to produce. A number of observations may be cited. Orias (5) and Coelho and Rocheta (6) noted that heart failure may occur following myocardial infarction from coronary ligation, although ventricular fibrillation was most likely to occur. The rôle of anoxia and shock (7, 8) have been emphasized as important factors leading to myocardial weakness. Coelho and Rocheta (9) employed a novel method of inciting myocardial damage and failure. They injected a solution of silver nitrate into the wall of the left ventricle, producing intense pulmonary edema and apparent heart failure. Cataldi (10) later noted the same when he injected silver nitrate into the wall of the right ventricle. These observations do not appear to be conclusive because of the intense toxicity of silver nitrate for many tissues, including the lungs. Armstrong (11) reported the occurrence of heart failure in rabbits following the introduction of highly irritating substances into the pericardiac sac. Bright and Beck (12) found that failure occasionally resulted from trauma to the heart. Their criteria for heart failure were not sharply defined. Furthermore, cardiac trauma frequently causes aberrant rhythms (13), with cardiac dilation, venous hypertension and a decrease in arterial pressure, in the absence of serious cardiac damage. Harrison and his co-workers (14) were able to produce heart failure in dogs by the use of either chloroform or potassium salts. Their procedures gave rise to certain features of myocardial insufficiency, but failed to clarify the precipitating factors.

In the course of a study of pulmonary edema of cardiac origin, it became necessary to devise a means of provoking heart failure in dogs with intact circulation, without the use of drugs other than for anesthesia. It was thought that cardiac damage of a more general distribution than that following occlusion of a coronary artery might lead more constantly to frank failure of the heart muscle. This report concerns the description of such a method, and its effects on the circulation.

Received for publication April 29, 1948.

¹ This study was aided by a grant from the Life Insurance Medical Research Fund.

² Fellow under the Life Insurance Medical Research Fund.

METHOD

Dogs of 4.8 to 15.4 kilograms weight were used. Following anesthesia with sodium pentobarbital, artificial respiration was applied through a tracheal cannula. The thoracic cage was opened widely by splitting the sternum, rendering the heart readily accessible. Small pericardial incisions were made over the left ventricle and over the right atrial appendage. When hemostasis had been secured, the animals were given 30-50 mgm. of heparin intravenously. Arterial blood pressure was measured from the right common carotid artery using a mercury manometer. Right intra-atrial pressure was recorded by means of a water manometer connected to a glass cannula tied into the atrial appendage; the base line was taken as the level of the orifices of the venae cavae. The heart rate was determined at frequent intervals.

In order to produce diffuse and graded myocardial damage, a 3.3 per cent suspension of potato starch (Mallinckrodt) in physiological saline was used. Such material had previously been used by Binger and his co-workers (15) in studies of pulmonary embolism. The greatest diameter of the starch particles ranges from 12 to 72 micra, and the particles are largely ovoid in shape. The 3.3 per cent suspension of starch contains approximately one million granules per cubic millimeter. After thorough shaking, 1 to 4 cc. of the suspension were injected rapidly through a hypodermic needle into the left ventricular cavity. During the injection, the aorta was occluded with the fingers immediately above the heart. Aortic occlusion was maintained for another two to three seconds after the injection was completed. By means of this technique it was expected that most, if not all, of the injected particles found their way into the coronary arteries. Injection of the starch suspension was repeated at one to seven minute intervals as desired.

Where the effects of increased blood volume on the damaged heart were to be investigated, a large 'donor dog' was anesthetized and heparinized. A carotid artery of the 'donor' was connected to a femoral vein of the recipient dog. Flow of blood was measured by means of a Ludwig's stromuhr introduced into the connecting tube; speed of flow could be controlled by a screw-clamp.

Following the experiments, the hearts were removed from the body, rinsed free of blood and weighed.

RESULTS

Myocardial Damage by Coronary Embolization. Using 10 animals, 1 to 4 cc. of starch suspension were injected into the left ventricle at intervals of two to seven minutes. In all instances, white mottled areas, about 1 mm. in diameter became visible under the epicardial membrane of both left and right ventricles. The first two or three injections of suspension did not influence right auricular pressure. The arterial blood pressure varied in response: a rise or fall of 15 mm. Hg mean pressure was frequently noted; in several instances there was no significant change. Except for transient premature ventricular contractions, the heart rate was not observed to change.

The effects of subsequent starch injections varied with the individual animal. In 7 dogs, definite myocardial insufficiency occurred from such treatment, although the events leading to failure were not uniform. With 4 of the animals, 1 to 5 additional injections of the suspension (2 to 11 cc.) resulted in a fall of arterial blood pres-

sure of 10 mm. Hg or less; in the remaining three animals a rise of 5 to 15 mm. Hg occurred. In the 7 experiments, the right intra-auricular pressure gradually increased by 15 to 30 mm. of water in 15 to 35 minutes. There was no significant change in heart rate. Each of these experiments showed a sudden change in cardiac performance following the next (4th to 9th) injection of 1 to 3 cc. of suspension.



Fig. 1. *Upper:* Two PHOTOMICROGRAPHS of the myocardium, the small arteries and capillaries of which contain starch granules. Section stained with iodine. *Lower:* photomicrograph of lung of a dog following production of acute congestive heart failure from coronary embolization with starch suspension. Congestion and moderate edema of the lung is apparent. Giemsa stain. $\times 125$.

Progressive cardiac dilatation occurred. Right auricular pressure rose 15-40 mm. water in 1 to 3 minutes, reaching a figure of 40 to 70 mm. water above the control level. Pulmonary edema ensued (engorgement, with numerous fine râles). There was then usually an associated decrease in heart rate, although heart action remained regular. Thereafter blood pressure fell to 25-40 mm. Hg. However, the rapid and striking increase in auricular pressure, dilatation of the heart, and pulmonary edema always appeared at a time when arterial blood pressure was still well elevated (80 to 95 mm. Hg).

In 5 of the 7 animals, the heart was so extensively damaged that these manifestations of heart failure caused death in one to five minutes. The hearts were enormously dilated, the rate slow, and the lungs filled with fluid. The right auricular pressures remained elevated, even after all cardiac activity had ceased. In two instances, pulmonary edema and cardiac dilatation gradually subsided, although right auricular pressure remained greatly elevated. Additional injections of 2 cc. of starch suspension were necessary to accentuate heart failure ending in death.

Table 1 summarizes the number of injections and total quantity of starch suspension required to provoke cardiac dilatation and pulmonary edema. It is noted that 10.0 to 21.7 cc. of the suspension per 100 grams of heart muscle were necessary to cause these results.

In 3 of the 10 experiments in this series, embolization of the coronary tree failed to provoke congestive failure. The introduction of 1 cc. of starch suspension produced a precipitous fall of blood pressure in one experiment. Two dogs exhibited a gradual fall of arterial tension following myocardial injury, with no rise in venous tension.

Myocardial Injury from Coronary Embolism Followed by Infusion of Blood. Eight dogs were used employing the technique described before. Each of the animals received a total of 2.5 to 8 cc. of starch suspension, divided into 1 to 4 injections at intervals of one to four minutes. No significant changes in arterial blood pressure or heart rate were noted following these injections, although in two experiments, the right auricular pressure rose 20 to 30 mm. of water. Following the last starch injection, the dogs were infused with 100 to 350 cc. of blood from the donor animal in a period of two to seven minutes. Neither cardiac dilatation nor pulmonary edema occurred; however, an increase of systemic blood pressure of 10 to 35 mm. Hg invariably occurred and right auricular pressure usually further increased 5 to 20 mm. of water. Thereafter, additional damage was inflicted on the heart by embolism, or further blood infusions were given. The procedures were repeated a variable number of times.

In five experiments, the injury to the heart, together with blood infusion, resulted in a striking elevation of auricular pressure (45 to 130 mm. water above control level), decrease in blood pressure and progressive cardiac dilatation and edema of the lungs. Table 1 summarizes these experimental data. The manifestations of acute heart failure persisted for 2 to 10 minutes. Death invariably occurred.

Myocardial damage and blood infusion did not produce such changes in three of the experiments. In these instances there was a continued fall of blood pressure following myocardial insult which persisted in spite of large infusions of blood. The animals showed no rise of right atrial pressure nor other signs of insufficiency of the heart.

Myocardial Insufficiency from Initial Infusion of Blood Followed by Coronary Embolism. Using 12 dogs, the experiments were begun with the continuous infusion of 100 to 300 cc. of blood over a period of one to six minutes. This abrupt increase of blood volume produced a rise of systemic arterial tension of 20 to 60 mm. Hg, and an increase of right atrial tension of 5 to 25 mm. of water. After completion of the infusion (1 to 7 minutes), starch suspension was injected (1 to 3 cc.) into the left ventricular cavity. No significant changes were observed in cardiac performance.

TABLE 1. SUMMARY OF EXPERIMENTS

DOG NO.	WT.	HEART WT.	STARCH REQUIRED TO PRODUCE C.D. AND P.E. ¹			DOG NO.	WT.	HEART WT.	STARCH AND BLOOD REQUIRED TO PRODUCE C.D. AND P.E. ¹				
			No. inj.	Total vol.	cc./100 gms heart				Starch			Blood	
									No. inj.	Total vol.	cc./100 gm. heart	No. inj.	Total vol.
Group I						Group II							
	kgm.	gm.		cc.			kgm.	gm.		cc.			cc.
1	7.3	51	4	5.5	10.0	8	7.8	51	2	4.0	8.0	2	200
2	10.2	58	5	9.0	17.2	9	6.8	62	5	8.0	12.9	2	200
3	6.8	68	6	9.0	13.2	10	8.0	53	4	6.0	11.3	2	350
4	15.4	138	6	14.0	10.1	11	13.0	88	5	10.0	11.3	2	350
5	7.5	69	9	15.0	21.7	12	8.6	61	4	8.0	13.1	3	300
6	9.5	70	8	13.0	19.9								
7	8.4	60	4	8.0	13.3								
						Group III							
						13	6.8	59	1	1.0	1.7	2	200
						14	7.3	65	3	3.0	4.6	3	300
						15	7.3	53	2	2.0	3.7	2	250
						16	4.8	37	1	1.5	4.0	2	300
						17	8.6	80	4	8.0	10.0	3	365
						18	9.8	68	2	5.0	7.4	2	275
						19	8.4	75	3	7.5	10.0	1	150
						20	8.9	58	1	2.0	3.5	3	300
						21	8.0	48	5	9.5	19.7	3	300
						22	6.8	46	4	8.0	17.3	2	400

Group I: Repeated injections of 3.3% starch suspension. *Group II*: Initial injection of starch suspension, followed by alternating injections of blood and starch suspension. *Group III*: Initial injection of blood followed by alternating injections of starch suspension and blood.

¹c.d. = Gross cardiac dilatation; p.e. = pulmonary edema.

TABLE 2. PROTOCOL

TIME	STARCH SUSP.	BLOOD	ART. PR.	RT. AUR. PR.	RATE PER MIN.	C.D. ¹	P.E. ²
<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mm.Hg</i>	<i>mm.Hg</i>			
0			110	30	150		
0-2		150					
2			140	50			
4			130	50			
4.5	2						
5			140	60	126		
7	2.5						
9			150	65	144		
10	3						
11			120	180	80	+++	+++
12			160	140	111 ³	+++	+++
14			150	120	132	+++	+++
16			90	110	112	++	++
17		100	80		100	++	
			0	20			

Dog no. 19, wt. 8.4 kgm., heart wt. 75 gm.

¹c.d. = Gross cardiac dilatation. ²p.e. = Pulmonary edema. ³Occasional ventricular extrasystoles.

Subsequent infusions and starch injections in 10 of the animals resulted in a gradual rise in right auricular pressure of 40 to 50 mm. water. The rise in auricular pressure was associated with decrease in heart rate, pulmonary edema and cardiac dilatation. The arterial pressure usually fell only slightly, and in several instances remained above control level for several minutes. In five experiments, dilatation of the heart and pulmonary edema subsided, although right atrial tension remained elevated. It is of interest that the majority of these 10 animals required much less starch suspension for the production of cardiac dilatation and pulmonary edema than did the animals in the former two groups. Table 2 represents a typical experiment.

Two of the dogs in this series did not develop congestive failure. One showed a rapid fall of blood pressure following myocardial injury. The other received large infusions and as much as 8.5 cc. of starch suspension. Possibly an additional blood transfusion might have resulted in heart failure.

DISCUSSION

From these experimental data, it is evident that heart failure in animals with intact circulation can be produced with considerable constancy by damaging the heart by coronary embolism with starch granules. Myocardial incompetence was judged by the criteria of elevated right auricular pressure, dilatation of the heart, congestion of lungs and liver and of pulmonary edema. On the other hand, Orias (6) and others (7, 16) have noted that local damage from ligation of coronary arteries only occasionally produces congestive failure. In our experience it has occasionally been possible to ligate the three major coronary arteries, leaving only small visible segments of normal heart muscle, without causing heart failure. Such treatment usually leads to a precipitous fall in blood pressure, or to ventricular fibrillation.

In a few of the experiments here reported, a fall of blood pressure, without rise in right auricular pressure, was also encountered. In view of the fact that cardiac output decreases after coronary ligation, even when the arterial pressure changes very little (19), it is probable that the acute hypotension observed by us was associated with a reduction in a cardiac output. Whether this reduction was primarily of cardiac (left ventricular) origin, or was due to an increase in the capacity of the peripheral vascular bed, has not been determined.

Starr and his associates (17) observed the absence of congestion following severe burning of the right ventricular muscle, although a rise in venous pressure was noted terminally in some instances. While heat applied directly to the heart may destroy large masses of the heart muscle, a surprising amount of the musculature may be spared. Moreover, an increase in capacity of the peripheral vascular bed, resulting in decreased venous return, was not ruled out in their experiments.

The damage inflicted by coronary starch embolism is diffuse, and involves the total myocardium. The extent of injury to the muscle fibers is difficult to judge histologically because insufficient time was allowed for anatomic changes to develop. It would seem that generalized and spotty injury to the heart muscle is more deleterious to the function of the heart, than massive but localized damage. It is of interest that a fall in blood pressure or aberrant rhythms, which are common with the production of massive infarcts, occurred only infrequently in the present series of experi-

ments. Congestive failure could generally be produced with less starch suspension when the blood volume was first augmented by 100 to 250 cc. of blood. This quantity of blood, in the absence of cardiac damage, produces a small rise of arterial pressure and an elevation of right auricular pressure of not more than 20 mm. of water. The fact that increased work imposed upon the damaged heart facilitates the development of failure is in agreement with the recent work of Landis *et al.* (18). They stimulated the muscles of the four extremities of the anaesthetized dog; when the heart had been damaged by ligation of a coronary artery, the 'exercise' resulted in a rise of central venous pressure lasting as long as activity was continued.

The important difference between the experiments of Landis *et al.* (18) and our experiments is that whereas coronary ligation alone produced a fall of venous pressure, coronary embolization produced a progressive rise in pressure. Also, even during exercise, infarcted hearts showed only a moderate increase in right auricular pressure as evidence of myocardial incompetence. In our experiments with starch, other manifestations of congestive failure eventually developed. It is possible that the fall in cardiac output which may follow coronary ligation (19) with an 'unloading effect' upon the damaged heart muscle, may occur only to a minor degree after starch embolization.

The circulatory changes noted in these experiments are in accord with accepted concepts concerning the functional responses of heart muscle in preparations with controlled inflow and peripheral resistance (22, 23). These concepts may be restated, simply, as follows. As muscle elements are damaged, remaining intact fibers lack the force to eject all of the inflowing blood. The residual ventricular blood, added to that from the auricles, increases the ventricular diastolic volume. Myocardial compensation occurs when the discrepancy between inflow and output becomes adjusted through greater force exerted by the lengthened muscle fibers. Intraventricular pressure at the end of diastole is increased, and consequently, intra-auricular pressure. Such changes may occur even when the venous return is diminished. With further myocardial damage, the remaining muscle fibers continue to fail in systolic discharge, the heart continues to dilate, and auricular pressure continues to rise. Essentially, such a sequence was observed in our experiments, and this progression of events in the failing heart of the dog with intact circulation appears to conform to the concepts of Starling (22). Other observations on experimental animals with intact and 'controlled' circulation (23) are in agreement with those of the Starling heart-lung preparation.

The introduction of right heart catheterization techniques in man has permitted a more accurate evaluation of cardiovascular performance in health and disease. Recent observations of the circulation in man (21) have suggested that changes in the magnitude of stroke volume may not be determined by corresponding changes in right auricular pressure, and that the concepts of Starling may not be applicable to human cardiodynamics. It is true that under the condition of the heart-lung experiments, increase in diastolic volume is accomplished by raising right auricular pressure. Furthermore, other observations on animals with intact circulation (23) and on man (24) indicate that an increase in ventricular diastolic filling may occur through a rise in right auricular pressure. However, Stead and Warren (21) suggest that the

cardiac output in man, under several conditions, may be significantly elevated as shown by the direct Fick procedure, although the mean right auricular pressure is 'within normal limits'. In their procedure, right auricular pressure was measured by a saline manometer attached to the intracardiac catheter. It is important, however, that normal atrial pressure (respecting atmospheric pressure) varies from -27 to $+27$ mm. of water (25), so that a latitude of more than 50 mm. of water tension may occur in any such experiment. Furthermore, unless special precautions are taken, a water manometer connected to an atrial catheter records pressures only during inspiration (26). A number of the subjects studied by Stead and Warren (21) had severe anemia or were submitted to exercise. With increased depth of respiration in both conditions, increased negativity of intrapleural pressure occurred with the possibility that misleading atrial pressure recordings may have been obtained. In short, the effect of decreased intrapleural pressure may be so great that a fall of atrial tension during inspiration is observed, whereas the 'effective' auricular pressure actually rose (24), together with cardiac output.

The resistance to flow between auricle and ventricle is so small that very slight increments of right auricular pressure have pronounced effects upon ventricular filling. In the heart-lung preparation, an increase in right auricular pressure of only a few mm. of water may double the stroke volume (22a). The output is usually less than the output of the heart of the intact animal, possibly because of the poorer condition of the muscle of the heart-lung preparation. However, the phenomena probably do not differ in any qualitative way.

It is possible that there are instances where accurately recorded right auricular pressure does not change, although stroke volume is increased. This situation can occur under only two conditions. The first is that ventricular diastolic volume increases without a rise of intraventricular pressure, which may be described as a decrease in diastolic 'tonus' (27). With energy output per unit fiber length and mechanical efficiency remaining the same, an increased amount of blood would be ejected per stroke (Starling's law). The second condition is that with no change in diastolic volume a larger volume of blood is ejected with each beat. This condition presumes that normally a considerable quantity of blood remains in the ventricle after systole, and that either the energy output per unit fiber length or the mechanical efficiency has increased. A change in diastolic tonus has been demonstrated experimentally in very few instances, notably after large doses of adrenalin (28). As to the second condition, an increased output of mechanical energy is known also to occur under the influence of adrenaline. However, it is questionable whether the residuum of blood in the ventricle, at least in the normal heart, is sufficient to account for an appreciable increase in stroke volume.

No conclusive experimental evidence is available indicating that one or both of the conditions mentioned operate in the human heart.

SUMMARY

A method has been described by which congestive heart failure can be produced in dogs with intact circulation with a considerable degree of constancy. The method

consists in the injection of a 3.3 per cent suspension of starch particles in physiologic saline into the left ventricular cavity, while the aorta is temporarily obstructed.

Repeated injections of this suspension resulted first in a progressive rise of right auricular pressure. This change was followed by dilatation of the heart, congestion of the lungs and the liver, and by pulmonary edema. The arterial pressure did not fall significantly until after the full blown picture of congestive heart failure had made its appearance. The experiments usually terminated in death.

Congestive heart failure could generally be produced with less starch suspension, when the blood volume was first augmented by 100-250 cc. of blood. In a few animals, congestive heart failure did not develop, but a precipitous fall in arterial pressure was observed, leading to death.

These results are discussed, and a critical survey is made of the changes in cardiodynamics which will affect ventricular filling and cardiac output.

The authors are indebted to Dr. H. L. White, Department of Physiology, for many helpful comments.

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JOINT REFLEXES AND REGULATION OF RESPIRATION DURING EXERCISE¹

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THE relation between muscular exertion and increased depth and rate of respiration is common knowledge. Whether this relation is neural or chemical is still not clear. In 1888 Geppert and Zuntz (1) showed that hyperpnea could be produced by tetanizing the hind limbs of animals whose spinal cords had been severed. The classical experiments of Haldane and his collaborators (2) led to the conclusions that chemical changes in the blood stream are the most important factors regulating the rate and depth of respiration. Nielsen (3) concluded that the increased pulmonary ventilation during severe exertion is the result of increased excitability of the respiratory centers toward CO₂.

Recently doubt has been cast on the concept of chemical control. Harrison *et al.* (4) and Comroe and Schmidt (5) concluded that reflexes from limbs play a part in the hyperpnea of exercise. The latter workers reported that passive flexion results in hyperpnea in men, dogs and cats and indicated that in animals the reflexes arise largely or entirely in and around joints and not in muscles or tendons. Other investigators (6-8), from studies of human subjects, arrived at essentially similar conclusions. Comroe (9) minimized the rôle of CO₂ and emphasized the importance of reflex factors. Gray (10) stated that limb reflexes are one of the most important factors in the regulation of respiration. He indicated that these reflexes originate in muscles, citing as reference the review by Comroe (9) who, with Schmidt (5), had concluded, however, that such reflexes originate in joints and not in muscles or tendons.

The viewpoints expressed in the preceding six references have not gone unchallenged. Asmusen and Nielsen (7) reported that limb reflexes are important only during light work. v. Euler and Liljestrand (11) found that even after section of the spinal cords of cats and dogs muscular work induced by electrical stimulation of either the distal part of the cord or of the hindlimbs was accompanied by a corresponding increase in ventilation. An almost similar effect was obtained after vagotomy and sinus denervation. Therefore they urged the necessity of a return to the hypothesis of direct chemical stimulation of the respiratory centers.

Recently the innervation of the knee joint has been studied from anatomical and physiological viewpoints (12, 13). In view of the importance of the general field of respiratory regulation, and of the possible importance of reflexes originating in joints in such regulation, the nerves to the knee joints of cats and dogs have been studied from the standpoint of their rôle in respiratory mechanisms.

METHODS

The distribution of nerves to the knee joints of dogs was determined by dissection. Six medial and 6 posterior nerves were removed, fixed in 10 per cent formalin

Received for publication May 10, 1948.

¹ Aided by a grant from the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service.

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and sections cut at 10 microns. These were stained either by the Bodian activated protargol method for axis cylinders or by a modified Masson's method for myelin sheaths. The myelinated and nonmyelinated fibers were counted in each nerve.

A variety of methods were used to record respiration. In a number of experiments, the trachea was cannulated and respiration recorded by means of an O₂ filled spirometer. It was found early in the course of this study that respiratory changes during movements of the legs were minimal so long as trauma was avoided. In many of the experiments, therefore, in order to avoid the trauma of inserting a tracheal cannula, other recording methods were used. For example, wire strain gages, connected as a Wheatstone bridge, were mounted on a strip of brass which was clamped at one end and placed across the thorax or abdomen. As the brass strip bent with respiratory movements, the gages were deformed. Their resistance, and therefore the current flow through them, was altered. The resulting bridge imbalance was either amplified and recorded with an inkwriter or else led directly to a galvanometer of a geophysical oscillograph.³ With proper precautions, this method gives an excellent indication of depth as well as rate of breathing. So long as the strip of brass is placed over the area of maximum excursion, usually the anterior abdominal wall, recorded changes in depth compare well with records obtained with the spirometer (fig. 1). There is a disadvantage in that if limb movements are transmitted in any degree to the trunk, they cause vibrations in the brass strip and irregularities appear in the records. If the interference was marked, it was avoided by using a recorder consisting of a face mask, in the airway of which a thermocouple was placed. Potential changes resulting from temperature differences in inspired and expired air were either amplified and recorded with an inkwriter or led directly to a geophysical galvanometer. This method, however, did not give an accurate index of changes in depth of respiration.

Blood pressure was recorded by means of a strain gage manometer (Statham Pressure Transmitter). This was connected to the right common carotid artery; 5 per cent sodium citrate was the anticoagulant. The output of the manometer led directly to a geophysical galvanometer.

The following experiments were carried out on cats and dogs anesthetized with sodium barbital or sodium pentobarbital, and occasionally with ether.

1. *Effects of Passive Flexion.* a) *Normal animals.* Ten cats and 25 dogs were used. Their legs were passively flexed at rates of 150 to 300 per minute. The experimenter held the limbs at the ankles with one hand and with the other hand steadied the hips. Beyond this, no special attempt was made to prevent movements from being transmitted to the trunk. The method made it possible to detect changes in muscle tone in the moving limbs. In many of the animals, other stimuli were used. These included movements of the trunk brought about by pushing on the hips in such a way as to mimic transmission of movements during passive flexion;

³ A geophysical galvanometer is a rugged instrument, usually of the moving coil type, which is available in a wide range of sensitivities. Banks of such galvanometers incorporated into compact, photographically recording oscillographs are ideal for many types of multiple recordings (16). A Hathaway oscillograph, which was used in the present experiments, contained galvanometers sensitive enough to be activated directly by strain gage and thermocouple pick-up units.

trunk movements were also produced by rubbing the sides of the thorax; the tracheal cannula was pushed to and fro through a very short distance; cut skin and muscle edges were rubbed or massaged. All these stimuli were carried out for periods of one minute. In some of the animals, the experiments were repeated after section of the right vagus nerve. In 2 dogs, the right hind limb was amputated at the thigh, except for femoral vessels and sciatic nerve, and effects of passive flexion of the knee studied. In most of the experiments, there were intervals of minutes or hours between observations in order to record changes in rate and depth of respiration in animals at rest. *b) Decerebrate cats.* Four additional cats were decerebrated by

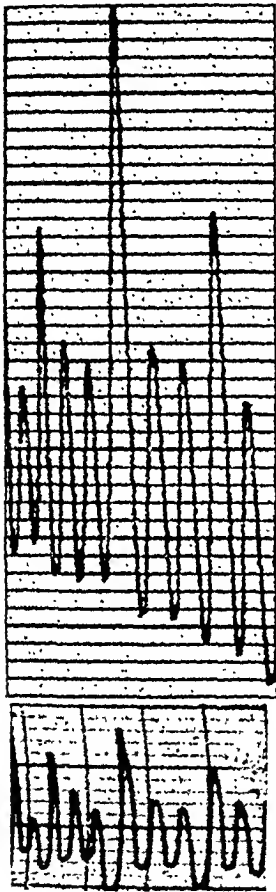


Fig. 1. *Upper*, O₂ FILLED SPIROMETER, record obtained from human subject who varied the rate and depth of his breathing. *Lower*, simultaneous recording using strain gage unit feeding into inkwriter. Note that the gage recorder affords a good index of changes in depth.

transection at the level of the superior colliculi after ether induction. The effects of passive flexion were studied about one hour after the decerebration. *c) Dogs with denervated knee joints.* In 4 dogs, the medial and posterior nerves to the knee joints were sectioned bilaterally under aseptic conditions. Five to 7 days later the effects of passive flexion were studied under conditions similar to those used with normal animals.

2. *Effects of Electrical Stimulation of Joint Nerves.* In 9 dogs and 3 cats, various articular nerves were exposed and placed on silver electrodes. Stimuli from a thyatron stimulator were applied at varying intensities. Frequency was also varied; four per second was most commonly used. In a number of experiments the duration

of individual stimuli was also varied. The period of stimulation was usually one minute. In 2 of the dogs, the action potentials of joint nerves were recorded photographically from a cathode ray oscillograph, and thresholds of the various components determined.

RESULTS

The distribution of nerves to the knee joint of the cat is known from previous work (12). In the dog there are at least two prominent articular branches. One of these arises from the saphenous nerve and accompanies blood vessels into the antero-medial region of the joint capsule. Some of its terminal branches pierce the capsule and enter the infrapatellar area. A nerve of similar origin, course and distribution is present in the cat. For purposes of simplicity, both are designated medial nerves. The other articular nerve in the dog is a branch of the tibial nerve. It arises in the popliteal fossa and enters the posterior region of the joint capsule. Occasionally there are two or more such branches. In the cat the corresponding nerve has a widespread distribution within the joint. Its larger fibers form Ruffini-type endings in the fibrous layer of the posterior region of the capsule. According to Sfameni (14) endings of this type occur in the corresponding region of the capsule of the knee joint of the dog. Because of their position relative to the knee joint, these are designated posterior nerves. Other articular branches undoubtedly exist in the dog, and probably arise from the femoral, obturator and peroneal nerves as they do in the cat, but no attempt was made to study them in this investigation. In the dog, the posterior nerve averages approximately 900 fibers of which 300 are myelinated. The medial nerve averages approximately 1400 fibers, of which 450 are myelinated. The shrinkage from the method of fixation and sectioning made an accurate determination of fiber sizes impossible. However, in each nerve the largest fibers were selected by inspection and their diameters measured. In all cases they were at least 11 to 12 microns in diameter. The shrinkage undoubtedly amounted to 10 or 20 per cent, so that the largest fibers in the articular nerves are more than 12 microns in diameter.

In the cat, the posterior nerve is the larger, and in neither of the two nerves do the number of nonmyelinated fibers exceed the myelinated. Fibers as great in diameter as 16 microns are present, but most of the myelinated fibers fall into two groups which range from 2 to 5 and from 7 to 10 microns in diameter.

Effects of Passive Flexion in Normal Cats (29 Observations in 10 Cats). Passive movements of one or both hind limbs produced a change in rate of respiration varying from -14 per cent to +17 per cent, and averaging $+2\frac{1}{2}$ per cent. Figure 2a shows the frequency distribution of these changes. In none of these experiments was there any significant change in depth. These results differ from those of Comroe and Schmidt (5) who found that such experiments in cats affected mainly the depth of breathing. The rate changes recorded in the present study are not great, and the fact that a decrease in rate was as apt to result as an increase indicates that the changes may not be the result of passive flexion. This is borne out by the fact that in animals at rest, the rate of respiration frequently varies as much as 10 per cent from one minute to the next. Furthermore, in dealing with the same animal, successive observations often showed considerable differences. Thus, in the first observation

in one particular animal, a 9 per cent increase in rate resulted; a 4 per cent decrease on the second; on the third and fourth trials, no change occurred.

Effects of Passive Flexion in Decerebrate Cats (21 Observations in 4 Animals). Passive flexion produced changes in rate of respiration varying from -7 per cent to +60 per cent, and averaging approximately +18 per cent. Depth often increased as well, so that the increase in minute volume was undoubtedly even greater. In all of the animals, extensor rigidity usually increased shortly after passive flexion was started. Frequently it became so marked that it was very difficult to flex the legs. These changes in muscular activity and in respiration often persisted for several minutes after stopping passive flexion.

Effects of Electrical Stimulation of Joint Nerves. Electrical stimulation of the

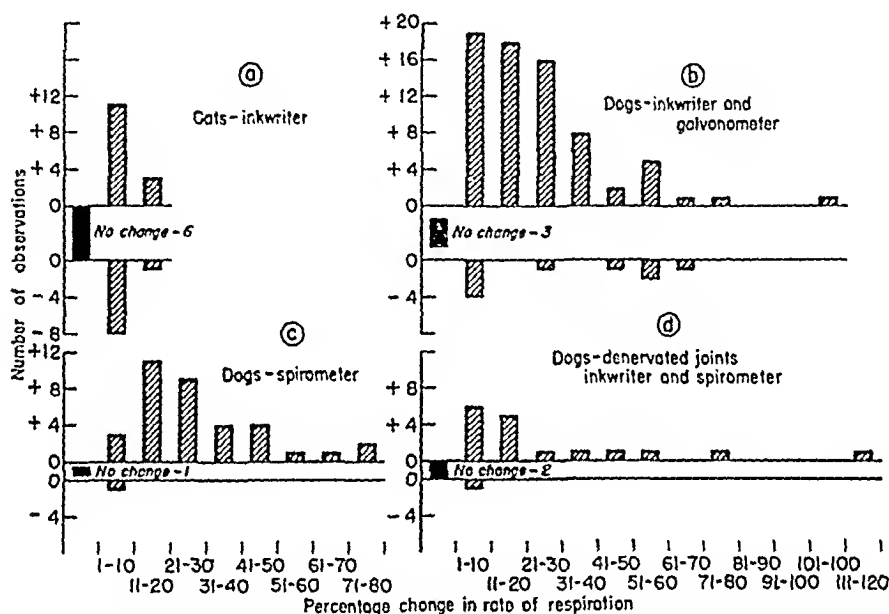


Fig. 2. SUMMARY OF CHANGES in respiration rate during passive flexion of hind limbs of cats and dogs.

posterior nerve at 4 volts caused a change in rate of respiration varying from no change to a 25 per cent increase, and averaging +8 per cent. The depth of respiration usually increased as well. When the intensity was decreased to 0.4 volts, the average rate increase was 3 per cent. There were 19 observations in 3 cats of the effects of the above intensities. At intensities less than approximately 0.4 volts, there were no observable effects on respiration. The threshold for large myelinated fibers, as determined by stimulation of muscle nerves, was approximately 0.02 to 0.03 volts.

Effects of Passive Flexion in Normal Dogs. The results were similar in character to those obtained in cats, but of greater magnitude. When respiration was recorded on an inkwriter or geophysical galvanometer, passive flexion was accompanied by changes in rate of respiration varying from -63 per cent to +105 per cent, and averaging +16 per cent (85 observations on 19 dogs). Figure 2b shows the frequency distribution of these changes in rate. Emphasis is placed on rate rather than depth changes because Comroe and Schmidt (5) reported that in dogs the respiratory re-

sponses to passive flexion are almost entirely increases in rate. In the experiments of the present study, changes in depth were extremely variable. At times there was little or no change (fig. 3); frequently it increased along with the rate; sometimes it increased when the rate decreased (fig. 4); occasionally it decreased when the rate increased (fig. 5E). In many of the dogs, there was enough reflex increase in muscle tone during passive flexion to make it difficult for the flexion to be carried out.

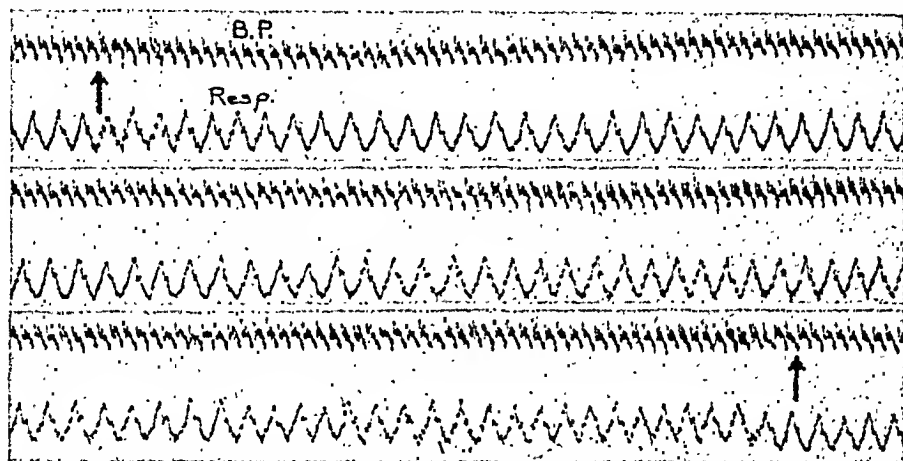


Fig. 3. RESPIRATION RECORDED with strain gage unit; blood pressure recorded with Statham Pressure Transmitter. Both units fed into geophysical galvanometers. This dog was in a period of hyperpnea (95/minute) which did not change during passive flexion for one minute (between arrows). Blood pressure (95/77) showed only a slight initial drop. The irregularities in the records are the result of transmission of movements from the flexion.

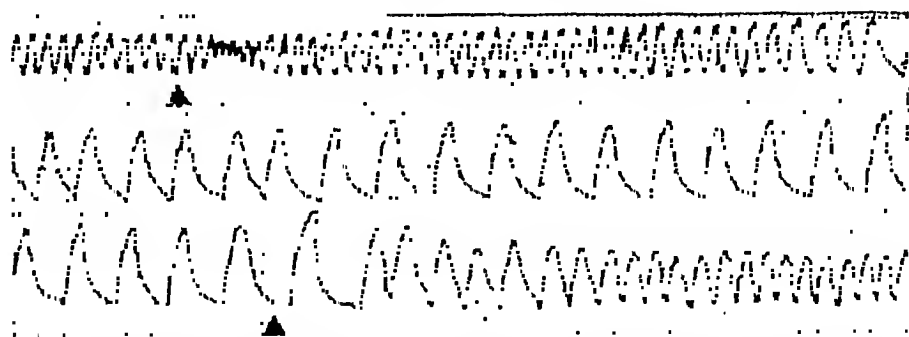


Fig. 4. RESPIRATION RECORDED with thermocouple unit feeding into an inkwriter. The dog was panting, but after passive flexion was begun (first arrow), respiration gradually increased in depth and decreased in rate and this persisted for a few seconds after flexion stopped (second arrow).

In animals at rest, the rate of respiration frequently varied considerably from one minute to the next. For example, the following rates per minute were recorded consecutively in a dog anesthetized with sodium pentobarbital: 12, 12, 12, 13, $12\frac{1}{2}$, $12\frac{1}{2}$, 13, 13, 15, $16\frac{1}{2}$, $18\frac{1}{2}$, $19\frac{1}{2}$, $18\frac{1}{2}$, $16\frac{1}{2}$, 15, $16\frac{1}{2}$, $14\frac{1}{2}$, 14, 14. These results indicate that changes supposedly induced by passive flexion could be partially or entirely the result of such spontaneous variations. In addition, in many animals, hyperpnea as great as 90 per minute (fig. 3 and 4) often appeared suddenly, persisted for varying periods of time, and as quickly disappeared. Similar periods of hyperpnea were observed by v. Euler and Liljestrand (11).

In 5 animals whose blood pressure was recorded during passive flexion, either no change occurred, or else there was an initial 5 to 10 mm. drop which persisted for but a few respiratory cycles (fig. 3).

Other stimuli were frequently as effective as passive flexion in altering respiration. For example, in an animal in which flexion caused a 105 per cent increase in rate, slight passively induced movements of the trunk for 1 minute caused an 82 per cent increase in rate.

In dogs whose respiration was recorded by means of a spirometer (37 observations on 12 dogs; 6 of these dogs had previously been studied when recording with an inkwriter), the following results were obtained. Passive flexion produced changes in rate of respiration varying from -10 per cent to +79 per cent, and averaging

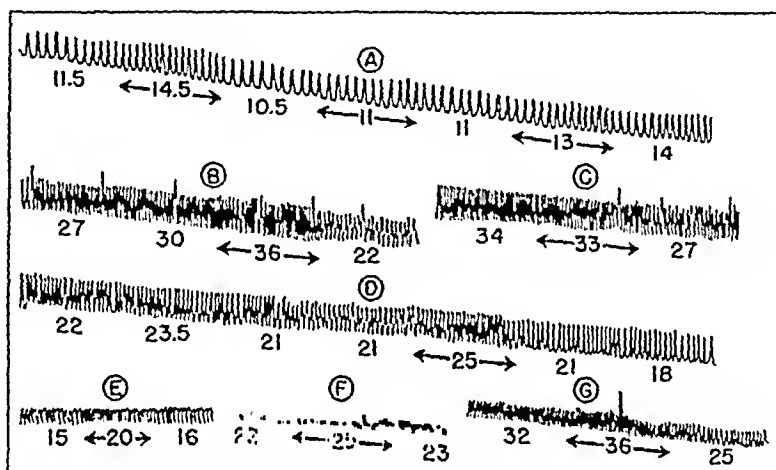


Fig. 5. All records except *E* obtained with spirometer and read from right to left. *E* obtained with inkwriter and reads from left to right. The numbers indicate rates per minute and intervals between arrows indicate stimulation for 1 minute. *A*, 3 periods of passive flexion of hind limbs of dog; only on third trial was there any increase in rate and depth. *B*, Passive flexion resulting in increased rate and depth which died away gradually. *C*, Same dog as *B*; passive movements of trunk caused increase in rate persisting for several minutes. *D*, Passive flexion resulting in rate increase; note spontaneous alterations in rate during periods of rest. *E*, Passive flexion resulting in increase in rate and slight decrease in depth. *F*, Passive flexion of amputated right hind limb of dog resulting in slight increase in rate. *G*, Same dog as *F*; cut muscles and skin edges in amputated stump massaged for one minute. Marked increase in rate which died away gradually.

+28 per cent. The frequency distribution of these changes is shown in figure 2c. Sometimes little or no change in depth occurred (fig. 5A) while at other times it increased considerably and, along with the increase in rate, often persisted after the passive flexion stopped (fig. 5B). The flexion undoubtedly was accompanied by a transmission of movement or vibration to the trunk. This may have been a factor contributing to hyperpnea, as indicated by figure 5C which shows the respiratory response to passively induced movements of the trunk for 1 minute. Slight movements of the tracheal cannula during the passive flexion may have been another contributing factor since in animals whose respiratory responses were marked, passively induced movements of the cannula produced similar responses, though of less magnitude.

Spontaneous changes in rate of respiration, and to a lesser extent in depth,

occurred in animals at rest (fig. 5D) just as they did in animals followed with the inkwriter or geophysical galvanometer.

The effects of sectioning the right vagus nerve were quite variable. Passive flexion carried out immediately afterwards were usually without effect on respiration. However, 30 to 60 minutes later changes similar to those of normal animals were usually obtained.

In 2 dogs, one hind limb was amputated at the thigh and the femur clamped so that flexion could be carried out without the transmission of movements to the trunk. The femoral vessels and sciatic nerve were left intact. The results of passive flexion were extremely variable although in general, if sufficient time elapsed after the amputation, increases in rate and sometimes in depth of respiration were obtained. Further experiments of this type were not carried out because of the difficulty of evaluating the effect of movement of cut skin and muscle edges in this type of preparation. Manipulation of any traumatized tissue may affect respiration markedly. Figure 5G illustrates the response to massage of cut muscles and skin in one of the amputated extremities.

Effects of Passive Flexion in Dogs with Denervated Knee Joints (20 Observations in 4 Dogs). In 4 dogs whose medial and posterior nerves had been sectioned bilaterally, passive flexion produced changes in rate of respiration varying from -3 per cent to $+111$ per cent, and averaging $+23$ per cent. The frequency distribution of these changes is shown in figure 2d. The denervation was not complete in any of these dogs since other, smaller branches undoubtedly reach the joint. In 1 dog, post-mortem examination showed that the posterior nerve to one of the joints had not been sectioned at operation.

The only complex endings consistently found in the knee joint of the dog and cat are Ruffini-type endings in the region of the capsule compressed during flexion, that is, the posterior part of the capsule. These endings are derived from the larger fibers in the posterior nerve. If joint movements are to have any reflex effects on respiration, it must be through stimulation of these complex and presumably proprioceptive endings during flexion, and yet the results in dogs whose posterior nerves were for the most part removed were scarcely distinguishable from those in normal dogs.

Effects of Electrical Stimulation of Joint Nerves. Stimulation of either posterior or medial nerves was effective only at fairly high intensities. Twenty-three observations in 9 dogs showed that stimulation at 4 volts caused a change in rate of respiration varying from -35 per cent to $+73$ per cent, and averaging $+16$ per cent. Depth of respiration usually increased whether or not the rate increased (fig. 6 and 7). Blood pressure nearly always dropped 5 to 40 mm., both systolic and diastolic, but returned to the previous level after a few respiratory cycles (fig. 6). In one instance, shortly after stimulation began, systolic pressure rose 27 mm. and diastolic 8 mm. Stimulation at 1.3 volts caused changes in rate varying from -2 per cent to $+38$ per cent, and averaging $+22$ per cent (five observations). There was much less change in depth of respiration and in blood pressure. Stimulation at 0.4 volts produced an average 12 per cent increase in rate (seven observations); 0.13 volts caused an average $5\frac{1}{2}$ per cent increase (nine observations). Reflex muscular contractions, whose frequency corresponded to that of the stimulating current,

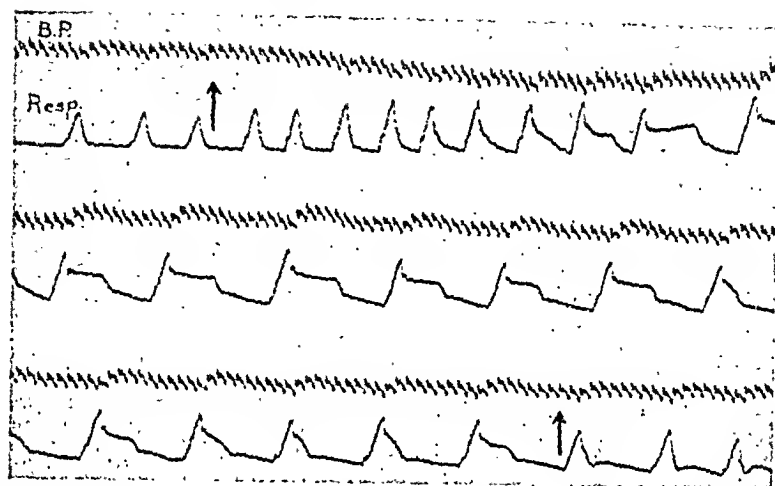


Fig. 6. SIMULTANEOUS RECORDING of respiration (strain gage unit) and blood pressure (Statham unit) on geophysical oscillograph during stimulation of both medial nerves of a dog. Four volts, 4 per second. Note initial increase in rate and depth and then a slowing with shift toward inspiratory side. Blood pressure (84/66) dropped about 20 mm. initially, but soon rose to previous level and respiratory waves became accentuated. In this animal, respiratory changes lessened in proportion to decrease in stimulus intensity.

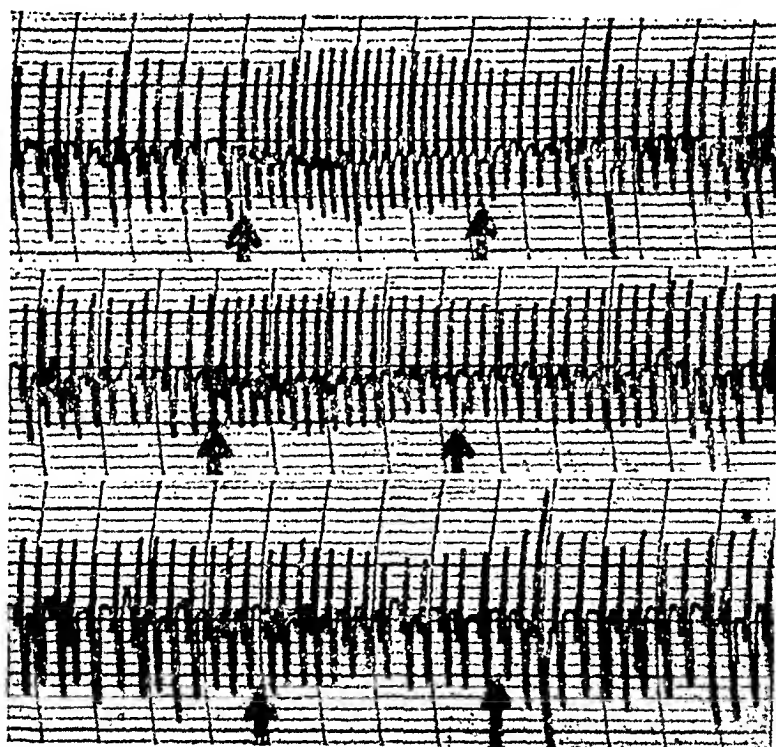


Fig. 7. RESPIRATION OF DOG recorded with strain gage unit and inkwriter. *Upper*: increase in rate and depth during stimulation of posterior nerve for 1 minute at 4 volts, 4 per second. *Middle*: 1.3 volts. *Lower*: 0.13 volts. The last voltage is still strong enough to stimulate larger fibers in the nerve. See figure 8.

usually occurred during periods of stimulation at the above intensities. There were a number of observations at intensities less than 0.13 volts, but in none of these was there any significant change in respiration.

The above observations included variations in frequency and duration of individual stimuli. For frequencies ranging from 4 to 100 or 200 per second, effects on respiration were similar. When the individual stimuli were of long enough duration to stimulate more of the slower conducting fibers (more than one half millisecond), effects on respiration were more pronounced than with shorter lasting stimuli.

It was evident from records of the action potentials of joint nerves that large fibers were stimulated and were conducting at intensities below those which caused changes in respiration. The threshold for the initial deflection in the action potential, an example of which is shown in figure 8, was 0.008 to 0.02 volts. Maximum height was reached at 0.04 to 0.13 volts. A second, smaller and more slowly con-



Fig. 8. ACTION POTENTIALS of posterior nerve. *Upper*, initial deflection maximal with stimulus of 0.04 volts. *Lower*, second and slower deflection begins at stimulus intensity of 0.06 volts. Sweep speed slightly faster in upper record.

ducting deflection appeared at 0.06 to 0.8 volts. Respiratory changes were noticeable only with stimulating voltages greater than those necessary to produce a maximum deflection of the faster conducting fibers.

Comparable electrical stimulation of the central ends of the cut saphenous, tibial and sciatic nerves yielded similar changes in respiration, but of greater magnitude, and in spite of deep anesthesia the animals frequently whined during the period of stimulation.

DISCUSSION

The respiratory changes in cats during passive flexion are quite small and can hardly be distinguished from spontaneous variations in animals at rest. Likewise, electrical stimulation of articular nerves was effective only at high intensities. These results indicate that under the conditions of experimentation, joint reflexes do not play a significant rôle in the regulation of respiration.

The respiratory changes in dogs were greater than those in cats, but still small and extremely variable in comparison with the consistent rise in ventilation which results from a small increase in alveolar CO_2 or during mild exercise. Comroe and Schmidt (5) reported an increase in respiratory minute volume in dogs during passive flexion and indicated that this change was almost entirely the result of an increase in rate. Their statement regarding these results is, however, somewhat ambiguous:

"Increase in respiratory minute volume was seen at least once in each experiment during passive movements of the legs; the increase varied from 22 to 125 per cent and averaged 52 per cent." This seems to mean that at times during each experiment no change or a decrease occurred. It is therefore difficult to compare their results with those of the present study, except in the following manner. In their experiments, no transmission of movement to the trunk occurred, whereas in the present study such transmission was usually allowed. It has been shown how this may affect respiration (fig. 5C). Nevertheless, the increase in rate of respiration which they reported was much greater than that obtained in this study. This indicates that some fundamental difference in method exists. A lead is offered by the fact that animals which were cannulated for spirometer recording were more responsive to passive flexion than animals whose respiration was recorded by other means. The fact that movements of the cannula or rubbing of cut skin edges frequently stimulated respiration indicates that any transmission of movement to the neck during flexion might similarly stimulate respiration. In Harrison's (4) and Comroe and Schmidt's experiments, such transmission did not occur, but other conditions were present which could have resulted in painful stimuli during flexion. The hind limbs of the animals had been amputated at the thighs and the femurs so clamped that transmission of movement to the trunk could not take place. There were, however, cut skin edges, cut muscles and connective tissue and these may well have been moved during flexion. Figure 5G illustrates the respiratory response to massage of cut muscles and skin in an amputated stump. Furthermore, painful stimuli may cause reflex muscular activity and thus an increase in metabolic activity. It cannot be emphasized too strongly that, so long as any nerve supply to the part distal to the amputation is left, neither the factor of pain nor reflex muscular activity can be ruled out.

There is no doubt, however, that definite respiratory responses to passive flexion do occur in the absence of trauma. Yet there is no evidence which indicates that joint reflexes produce these changes, because: Stimuli other than passive flexion, in particular those stimuli which were secondarily induced by the flexion, were effective respiratory stimulants. Similar changes occurred in dogs whose knee joints had been partially denervated. Electric stimulation of joint nerves yielded respiratory responses only at high intensities, while no changes occurred at voltages which were still effective for the myelinated fibers of a diameter range which includes proprioceptive fibers. The responses which do occur with electric stimulation seem best explained as a pain hyperpnea. The drop in blood pressure which occurs initially in painful stimuli (fig. 6) may well be partially responsible for a reflex hyperpnea. Figure 3 of Comroe and Schmidt's paper shows such an initial drop. Jaroschy (15) recorded respiration and blood pressure during faradic and mechanical stimulation of the capsule of the knee joint of the rabbit. There resulted a decrease in depth of respiration, followed by an increase and a slowing of rate. Blood pressure dropped initially and then rose. Finally, one must take into account spontaneous alterations in respiration which might make induced changes seem larger than they actually are. One can only conclude from these results that respiration tends to change in response to any stimulus to which the animal is subjected. Since it has been shown (1, 11)

that in cats and dogs, absence of afferent impulses does not interfere with the regulation of respiration during muscular activity, and in view of the present results, it is concluded that under the conditions of anesthesia commonly used, joint reflexes (or limb reflexes) do not play a significant rôle in respiratory regulation.

Barbiturate anesthesia so depresses the central nervous system, including the respiratory centers, that in deep anesthesia reflex mechanisms may be greatly suppressed. The effect on the spinal cord may resemble a cord transection. Therefore in order to settle the problem of the rôle of limb reflexes, conscious human subjects should be used. Even so, it may be difficult to interpret the results of experiments on such subjects. Passive movements, for example, produce relatively slight changes and there is no guarantee that psychic influences or reflex increase in muscle tone are ever eliminated. Experiments in which the effects of exercise plus ischemia are studied are probably not valid since this type of experiment is usually painful. Even if the subject learns to suppress the pain, it is doubtful that reflex effects can be eliminated. The factor of ischemic pain must be considered in explaining the results of Asmussen, Christensen and Nielsen (6) who found that when the circulation of exercising limbs was occluded, the O_2 consumption dropped, but the ventilation remained approximately the same. These same workers concluded that reflexes originating in the moving limbs were responsible for maintaining ventilation. They expected that the nerve impulses would reach the respiratory centers by ascending in the dorsal funiculi of the spinal cord. However, one of their subjects who suffered from *tabes dorsalis*, during voluntary and electrically induced work, had respiratory changes similar to those of normal subjects. Because of this they concluded that some other pathway must be present in the spinal cord by which impulses could reach the brain stem. Their assumption appeared to be that the characteristic lesion of *tabes* is destruction of the dorsal funiculi. Actually, the inflammatory and destructive processes of *tabes dorsalis* involve dorsal root ganglia and dorsal root fibers, and proprioceptive fibers are destroyed early in the course of the disease. Degeneration of dorsal funiculi occurs because central processes of dorsal root ganglion cells form a large portion of the funiculi. Other proprioceptive pathways, such as spino-cerebellar, do not function since impulses do not reach them, yet they show no degeneration since synaptic junctions are interposed which prevent degeneration from ascending. In *tabes dorsalis*, therefore, all afferent pathways are eventually affected, and proprioceptive pathways most severely. On this account, it is extremely difficult to see what the other pathways might be which Asmussen, Christensen and Nielsen postulated. The fact that in their tabetic subject respiratory adjustment to exercise was essentially normal indicates how doubtful it is that limb reflexes are important in the regulation of respiration. It also indicates the value of correctly interpreted clinical material in investigating this field of respiratory physiology.

There is as yet no convincing evidence which should cause us to discard the concepts of Haldane and his collaborators regarding the regulation of respiration according to metabolic needs. It would be very difficult to correlate limb reflexes with the actual events in exercise, particularly when pulmonary ventilation increases with increasing work, even though the rate of movement remains the same. The effect of limb reflexes would have to be an extremely powerful one to explain the

fact that in a man who runs 100 yards, the great ventilation and metabolic exchange which are necessary occur quite effectively afterwards in the absence of limb movements. This would indeed be a potent reflex, and yet one easily suppressed since little or no breathing takes place during the sprint.

SUMMARY

Passive flexion of the hind limbs of cats and dogs is accompanied by variable changes in rate and depth of respiration. These changes do not appear to result from the stimulation of receptors in the knee joint because: Other types of stimuli, including those secondarily induced by the flexion, such as movements of the trunk, were as effective in stimulating respiration. Similar changes occurred in dogs whose knee joints had been partially denervated. Electric stimulation of nerves to the knee joint was effective only at intensities great enough to activate slower conducting fibers, resulting in what appeared to be a pain hyperpnea. It is concluded therefore that, under the conditions of anesthesia used, joint reflexes do not play a significant rôle in the production of respiratory changes during passive flexion. It is also concluded that there is no satisfactory evidence which indicates that limb reflexes are important in the regulation of respiration during exercise in man.

We wish to express our appreciation to Drs. John Green and Norman Jarvik for their help and criticism during the course of the experiments, and we thank the following students for their aid in conducting the experiments: Bernard Agranoff, Nicholas Bash, Abraham Elson and James Morrish

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EFFECT OF ACID-BASE CHANGES ON EXPERIMENTAL CONVULSIVE SEIZURES^{1, 2}

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PROCEDURES which change the acid-base balance of the body are employed in the diagnosis and therapy of convulsive disorders. As examples the hyperventilation test used in the diagnosis of petit mal (1-5), and the use of fasting (6-9) and particularly the ketogenic diet (10-14) in the control of seizures may be cited. Experimental procedures used in the study of the physiology and pharmacology of seizures frequently produce incidental modifications of the acid-base balance. In a many-sided study of seizures in progress in this laboratory it became important to know to what extent these acid-base changes might affect the results obtained.

METHODS

Rats of the Sprague-Dawley strain were used. Convulsions were produced by a 60-cycle alternating current apparatus with current output independent of the external resistance. Spiegel corneal electrodes (15) were employed. The stimulus duration was 0.2 second. A slight clonic seizure, lasting 10 seconds or more, was chosen as the end-point in threshold determinations, and the current necessary to produce such a response was used as the measure of minimal electroshock seizure threshold. For observation of changes in seizure pattern, maximal seizures were produced by a current of 150 mA., 0.2 second duration, or about 7 times threshold for minimal seizures.

In order to determine the effect of modifications of the acid-base balance upon the threshold for seizures produced by convulsant drugs, a group of 30 rats weighing between 250 and 360 grams was given graded doses of Metrazol by subcutaneous injection. The number of animals convulsing was recorded. The threshold convulsive dose (ED_{50}) was calculated by probit analysis (16). The observations on Metrazol were repeated with another group of 20 rats weighing between 140 and 210 grams and having a higher threshold. The ED_{50} for picrotoxin was similarly established.

Metabolic acidosis was produced by ammonium chloride given by stomach tube in 1 M or 0.3 M solution. Metabolic alkalosis was produced by sodium bicarbonate

Received for publication June 7, 1948.

¹ This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

² A preliminary report of this work was presented before the American Physiological Society in Atlantic City, March 1948, *Federation Proc.* 7: 52, 1948.

or sodium acetate in similar concentrations. Control animals were given comparable volumes and concentrations of sodium chloride solution. Respiratory acidosis was produced by placing the rats in a glass jar through which flowed a gas mixture containing 89 per cent O_2 and 11 per cent CO_2 . Control rats were allowed to breathe 89 per cent O_2 and 11 per cent N_2 . The rats were shocked within 20 seconds after removal from the jar.

Blood samples were not taken from rats whose convulsive behavior was shortly to be measured, because the loss of blood might affect threshold or seizure pattern. Other rats concurrently subjected to the same disturbances of acid-base balance were therefore used. The blood was removed by heart puncture and handled anaerobically (17). The plasma pH was measured at room temperature by means of a standardized glass electrode. A correction of -0.014 pH units per degree was applied to correct the pH to $38^\circ C$. The total carbon dioxide concentration of the plasma was measured by the method of Van Slyke and Neill (18). Plasma bicarbonate concentration and pCO_2 were calculated using a pH of 6.10 and a carbon dioxide solubility coefficient of 0.0301. The results were plotted on a pH-bicarbonate diagram on which pH values were abscissae and plasma bicarbonate concentrations in mM/l were ordinates.

Previous work in this laboratory (19) has shown that normal rat blood taken by heart puncture has a pH of 7.41 and bicarbonate concentration of 24.3 mM/l, and that the normal buffer value of rat blood as determined by its carbon dioxide absorption curve is -24.7 mM per liter of plasma per pH unit. A straight line having this slope was drawn through the normal point on the pH-bicarbonate diagram. The vertical distance expressed in mM plasma bicarbonate between any observed point and the normal buffer line is a measure of the extra fixed acid or fixed base in the blood (20).

In experiments on rabbits cortical potentials in the restrained unanesthetized animals were led from epidural electrodes permanently implanted in the skull, and they were recorded with a Rahm electroencephalograph. Metabolic acidosis or alkalosis was produced by intravenous or intraperitoneal injections of ammonium chloride or sodium bicarbonate solutions. In some experiments single condenser shocks were given to one hemisphere, and the threshold for evoked transcortical potentials was measured (21, 22). In other experiments subcutaneous doses of Metrazol were given to produce subconvulsive slow-wave discharges (21).

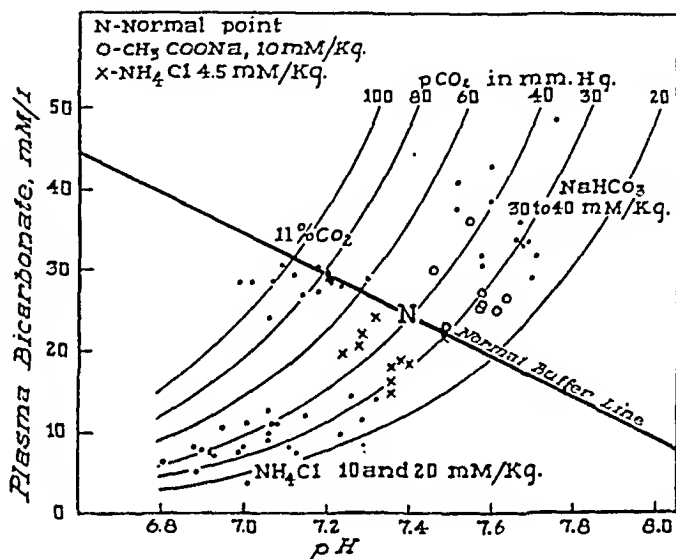
RESULTS

The acid-base changes produced are shown on the pH-bicarbonate diagram in figure 1. *N* represents the normal point, and the straight line is the normal buffer curve of rat blood. Doses of ammonium chloride between 10 and 20 mM/kgm. added an average of 24 ± 3 mM/l extra fixed acid, and doses of 4.5 mM/kgm. added 6 ± 1 mM/l extra fixed acid. Sodium acetate in doses of 10 mM/kgm. added 7 ± 2 mM/l extra fixed base, and sodium bicarbonate in doses of 30 to 40 mM/kgm. added 15 ± 1 mM/l extra fixed base. Breathing 11 per cent CO_2 raised the pCO_2 to an average of 88 ± 4 mm. Hg. Control rats given doses of sodium chloride comparable

to those of the other salts had a slight metabolic acidosis of about 3 mM/l extra fixed acid.

The effects of acid-base changes upon the electroshock seizure threshold of rats are shown in table 1. Untreated control rats showed a small rise in threshold when tested first in the morning and again in the afternoon. Rats given sodium chloride and having a slight metabolic acidosis had a greater rise in threshold. This rise can not be attributed to the metabolic acidosis, for rats having more extra fixed acid in the blood as the result of ammonium chloride administration had no increase in threshold. It has been shown in this laboratory (23) that an increase in extracel-

Fig. 1. A PH-BICARBONATE DIAGRAM of rat plasma. The straight line is the normal buffer curve of rat blood, and *N* is the normal point. Measurements on individual rats are represented. The experimental procedures to which the different groups were subjected are indicated on the figure.



lular sodium concentration in itself raises the threshold of rats. Consequently, the rats receiving sodium chloride, rather than those receiving no salt, are the appropriate control animals for those receiving alkalinizing salts containing sodium. The control groups with which the experimental groups are compared are designated by the Roman numerals in column five of table 1.

The results show that a moderate metabolic alkalosis significantly decreases seizure threshold, but this decrease is small. The same is true of severe metabolic alkalosis. Only the most severe degree of metabolic acidosis produces a change in seizure threshold. The increase (14%) is not so great as that which can be achieved by means of anticonvulsant drugs (22). Severe respiratory acidosis is without effect on seizure threshold.

The results of the observations of the effect of respiratory acidosis upon the ED_{50} of Metrazol and picrotoxin are given in table 2. They demonstrate that severe respiratory acidosis does not change the threshold convulsive dose of the drugs employed.

Normal rats given supramaximal shocks exhibit a seizure whose pattern and duration are relatively constant; the seizure is profoundly modified by anticonvulsant

TABLE 1. EFFECTS OF ACID-BASE CHANGES UPON ELECTROSHOCK THRESHOLD IN RATS

TREATMENT	N	ACID-BASE BALANCE	CHANGE IN THRESHOLD OVER PRIOR CONTROL	CHANGE CORRECTED FOR APPROPRIATE CONTROL	P
			%	%	
None (I)	11	Normal	$+2 \pm 1.8$		
NaCl (II)	24	Slight Metabolic Acidosis	$+7 \pm 1.3$		
NH ₄ Cl	12	Mild Metabolic Acidosis	$+2 \pm 0.6$	0 ± 1.9 (I) ¹	0.9
NH ₄ Cl	18	Extreme Metabolic Acidosis	$+16 \pm 1.1$	$+14 \pm 2.1$ (I)	0.01
CH ₃ COONa	11	Mild Metabolic Alkalosis	$+0 \pm 1.8$	-7 ± 2.2 (II)	0.01
NaHCO ₃	30	Severe Metabolic Alkalosis	$+2 \pm 0.9$	-5 ± 1.6 (II)	0.01
89% O ₂ (III)	14	Normal	$+3 \pm 2.9$		
11% N ₂		Severe Respiratory Acidosis	$+2 \pm 1.2$	-1 ± 3.1 (III)	0.6

¹ The Roman numerals in this column refer to the control groups in column 1 with which the experimental groups are compared.

TABLE 2. EFFECT OF RESPIRATORY ACIDOSIS ON THRESHOLD CONVULSIVE DOSE OF METRAZOL AND PICROTOXIN

DRUG	89% O ₂ PLUS	NO. OF INJECTIONS	E.D. ₅₀
	%		mgm/kgm.
Metrazol (large rats)	11 N ₂	53	30 ± 1
	11 CO ₂	57	30 ± 1
Metrazol (small rats)	11 N ₂	20	45 ± 1
	11 CO ₂	20	45 ± 1
Picrotoxin	11 N ₂	44	1.4 ± 0.1
	11 CO ₂	33	1.3 ± 0.1

drugs (24). In the present study severe metabolic acidosis or alkalosis failed to produce significant changes in the seizure pattern or in the duration of its components.

The resting electroencephalogram of rabbits was not affected by severe meta-

bolic acidosis or alkalosis. Subconvulsive doses of Metrazol produced typical episodes of high-amplitude slow-wave activity, but these were not affected by acid-base changes. The amount of Metrazol required to produce them was not altered. When condenser shocks were applied to one hemisphere and the response recorded from the other, no change in the threshold or pattern of cortical response was caused by acidosis or alkalosis.

DISCUSSION

The conception that alkalinity increases and acidity reduces excitability of neurones is not universally supported by observations at various levels of integration in the nervous system. Changes in pH brought about by variations in CO₂ tension may alter the excitability of peripheral nerve (25), but the effects are apparently minimal if CO₂ tension is held constant while pH is varied by other means (26). Increased CO₂ tension in the absence of pH changes in general tends to raise threshold and to prevent spontaneous firing in nerve, but it also increases the amplitude of after potentials and associated changes in excitability (26), an action which might conceivably promote the development of the multiple rapid discharges which are a characteristic feature of convulsive activity in the brain (27). As an illustration of the differences in responsiveness of central neurones, CO₂ can be shown to have a purely depressant action on spinal motoneurones (28), but it increases the discharges from the isolated inspiratory center of the medulla (29). In general, the sensitivity of various central neurones to depression by metabolic acidosis differs widely (30). It has been reported that metabolic or respiratory alkalosis increases spontaneous cortical electrical activity and decreases electrical threshold for cortical afterdischarge, while acidosis has the opposite effects (31). On the other hand, it has been emphasized that nearly fatal acid-base changes are required to produce EEG abnormalities (32). Some investigators have reported a protective effect of CO₂ inhalation against Metrazol (33) and strychnine (34) seizures. However, others have found that convulsive Metrazol discharges are singularly unresponsive to hyperventilation, CO₂ inhalation and intravenous injection of acid or alkaline solutions (35).

Among the convulsive disorders, it is those of the petit mal triad which tend to respond specifically to changes in acid-base balance, particularly to small changes in CO₂ tension (2, 36, 37, 38), although other seizure types or EEG abnormalities may be occasionally induced by prolonged hyperventilation (3, 5). Even among patients who might be clinically classified in the petit mal group, it is not always possible to precipitate seizures or EEG spike and wave formations by hyperventilation, nor to prevent them by CO₂ inhalation (39). Thus sensitivity to changes in acid-base balance is by no means of universal occurrence in convulsive disorders.

The present experiments indicate that seizures produced by electrical stimulation or by Metrazol or picrotoxin are similarly resistant to acid-base changes, and therefore suggest that modification of acid-base balance may be a relatively unimportant factor in the mechanism of action by which anticonvulsant drugs and other agents modify the properties of experimental convulsions.

SUMMARY

Mild metabolic acidosis and severe respiratory acidosis have no effect upon the electroshock seizure threshold of rats. Severe metabolic acidosis raises the threshold about 14 per cent, and mild and severe metabolic alkalosis lower the threshold about 7 per cent. Severe respiratory acidosis has no effect upon the threshold convulsive dose of Metrazol or picrotoxin.

Severe metabolic acidosis or alkalosis has no effect upon the maximal seizure pattern of rats, or upon the resting electroencephalogram, the subconvulsive Metrazol EEG discharge and the threshold for evoked cortical potentials in rabbits.

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A STUDY OF THE ADRENOTROPIC RECEPTORS

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THE adrenotropic receptors are those hypothetical structures or systems located in, on or near the muscle or gland cells affected by epinephrine. The concept of a receptive mechanism was introduced by Langley (1, 2) to explain the action of curare on skeletal muscle. Dale was probably the first to make significant use of the receptor concept in connection with the sympathetic nervous system. In his classical paper (3) on the sympatholytic action of the ergot alkaloids, he recognized that what he called the sympathetic myoneural junction could also be called 'the receptive mechanism for adrenaline'; and he used this mechanism to explain the fact that the ergot alkaloids prevented only the motor (excitatory) actions of epinephrine and had no effect on the inhibitory actions of epinephrine or on the excitatory actions of barium or pituitrin.

The adrenotropic receptors have been considered to be of two classes, those whose action results in excitation and those whose action results in inhibition of the effector cells. Experiments described in this paper indicate that although there are two kinds of adrenotropic receptors they cannot be classified simply as excitatory or inhibitory since each kind of receptor may have either action depending upon where it is found. The evidence for these conclusions is, in brief, that a series of six sympathomimetic amines has one order of potency—1, 2, 3, 4, 5, 6—on the following functions: vasoconstriction, excitation of the uterus and ureters, contraction of the nictitating membrane, dilation of the pupil and inhibition of the gut. In contrast, this same series of amines has an entirely different order of potency—2, 4, 6, 5, 3, 1—on the following functions: vasodilation, inhibition of the uterus and myocardial stimulation.

The variations in activity could be due to any or all of three factors: *a*) quantitative differences in potency, *b*) qualitatively different effects or *c*) differences due entirely to the experimental methods used. If the last two factors are controlled as much as possible by the selection of the amines and by using suitable experimental techniques, then the variations in activity are presumably due to actual differences in the receptors involved. Tentatively the first kind of receptor has been called the *alpha* adrenotropic receptor and the second kind the *beta* receptor. This concept of two fundamental types of receptors is directly opposed to the concept of two mediator substances (sympathin E and sympathin I) as propounded by Cannon and Rosenblueth (4) and now widely quoted as a 'law' of physiology. Results reported in this paper indicate that conclusions drawn by Cannon and Rosenblueth are open to controversy and that there is no known amine which fulfills the requirements for

Received for publication May 10, 1948.

either sympathin E or I. The evidence presented strongly supports the concept that epinephrine is the only sympathetic adrenergic mediator.

The amines used were restricted to those which produced responses in the same dosage range as epinephrine, for which equivalent doses had similar durations of action, and which had qualitatively identical actions on the myocardium. None of them produced tachyphylaxis. The following amines were studied (abbreviations given in italics will be used throughout the remainder of this paper):

- I. *dl*-(3,4-dihydroxyphenyl) ethanolamine. Arterenol, *art*.
- II. *dl*-(3,4-dihydroxyphenyl) isopropanolamine. Cobefrine, *methyl-art*.
- III. and IV. *dl*, and *l*-(3,4-dihydroxyphenyl) methyl ethanolamine. Racemic and levo epinephrine, *dl-epi*. and *l-epi*.
- V. *dl*-(3,4-dihydroxyphenyl) methyl isopropanolamine. *Methyl-epi*.
- VI. *dl*-(3,4-dihydroxyphenyl) isopropyl ethanolamine. *N*-isopropyl arterenol, *N-iso-art*.

All of these amines have been studied extensively in the past (5-20) and hence there is a voluminous literature concerning them. No previous studies, however, have included all of them together in a quantitative comparison.

PROCEDURE

Dogs, cats, rats and rabbits were used in this study. The amines, in the form of their hydrochlorides, were made up in M/1000 stock solutions using isotonic saline containing 0.1 per cent sodium bisulfite and 0.1 per cent chlorobutanol as the solvent. Higher dilutions were made at the time of use with isotonic saline. The use of other drugs was kept to a minimum. Sodium pentobarbital, 20 to 30 mgm. per kgm., or urethane, 1 gram per kgm., was used as the anesthetic. The dogs and rabbits were usually pretreated with morphine sulfate, 10 mgm. per kgm. Atropine, 0.5 mgm. per kgm., was used as the anticholinergic agent. Three sympatholytic agents were used: ergotoxine, 2 mgm. per kgm., dibenamine (21), 25 mgm. per kgm. and Priscol (22).

Two types of dosage were employed with the amines: equimolar and equivalent. Truly equivalent doses in intact animals were many times unobtainable. For example, in attempting to determine the equivalent dosages necessary to inhibit the intact intestine it was found that the marked cardiovascular effects (some pressor and others depressor) interfered with or in themselves produced changes in the intestinal activity. For this reason equivalent doses were determined chiefly on isolated structures and equimolar doses compared in intact animals. In determining the relative activity of equimolar doses, both the degree and the duration of the response were considered. The amines were administered intravenously unless otherwise stated.

Cardiovascular. There are at least three functions served by adrenotropic receptors in the cardiovascular system: vasoconstriction, vasodilation and myocardial stimulation. Arterial pressure changes in themselves are not suitable for comparing the activity of these amines on these receptors because the arterial pressure represents the resultant of a number of factors such as the cardiac output and venous return, the balance between constriction, dilation and blood viscosity, and the mechanical effects produced by the muscular contractions such as occur in the heart,

intestine or uterus. Therefore in order to study each type of receptor it was necessary to work with more or less isolated parts of the cardiovascular system and then to correlate these results with those obtained from arterial pressure studies.

The arterial pressure was recorded by means of either a mercury manometer or a Hamilton manometer (25) from the carotid or femoral artery. In many of the dogs the aortic pressure pulse was recorded by means of a high frequency Hamilton manometer using a metal sound passed through the left common carotid into the aortic arch. In these latter experiments two recording cameras were used, one running continuously at low speed for a complete record of the amine response, and the other at high speed for pulse contours at appropriate times during the amine response.

The blood flow was measured in the important vascular beds by means of flowmeters introduced into either the venous outflow or the arterial supply. A Shipley optically recording rotameter (24, 25) or a modified Soskin type (26) 'bubble' flowmeter was used. The 'bubble' flowmeter was equipped with a reversing system so that only a single air bubble was needed for any one experiment. Heparin, 10 mgm. per kgm. every two hours, was used as the anticlotting agent.

The effects of the amines on the vascular receptors were compared by determining their actions on the vasomotor resistance (VR). The VR represents the pressure-flow relationship calculated by the formula (27), $VR = \frac{P - 20}{F}$ in which P is the arterial pressure in mm. Hg and F is the volume flow in cc. per minute. $P - 20$ was used since the P/F ratio changes abruptly at a pressure of about 20 mm. Hg (28), a phenomenon due in part to the presence of the cellular elements in the blood.

The results on the VR of the renal, mesenteric and femoral beds are shown in figure 1 and tabulated in table 1. It will be seen that the increase in VR produced by *epi.* in these vascular beds bears an inverse relationship to the decrease in VR produced by *N-iso-art.* In the bed (renal) which shows the least dilation with *N-iso-art.*, *dl-epi.* is the most active among the racemic amines in producing constriction. In the femoral bed, in which *N-iso-art.* produces the greatest dilation, the net constrictor effect of *dl-epi.* is diminished by its own dilator action. These results are interpreted as showing 1) that the ratio of constrictor to dilator receptors varies in the different vascular beds and 2) that *dl-epi.* is the most active of the racemic amines on the constrictor receptor. *Art.* on the other hand, produces the greatest constriction in those beds which have the most dilator receptors, since this amine has very little action on the dilators (see below). The remaining two racemic amines show variable amounts of constrictor as against dilator activity.

This same relationship was shown in the arterial pressure responses to these amines as shown in figure 2. In the cat, which appears to have many dilator receptors, *art.* is the most active pressor agent among the racemic amines, with *dl-epi.* being third. In the rabbit, which appears to have few dilator receptors, *dl-epi.* is more active than *art.* Even *N-iso-art.*, which is a depressor in cats and dogs, produces a slight pressor response in rabbits. Blood flow studies showed that this pressor action of *N-iso-art.* in rabbits was due in part to vasoconstriction.

The comparative effects of these amines on the vasodilator receptors were deter-

mined by measurements of the coronary flow in perfused hearts (see below) and by their depressor effects in intact animals after the administration of the sympatholytic agent Dibenamine. In cats treated with the sympatholytic agent, *l-epi.* and *dl-epi.*

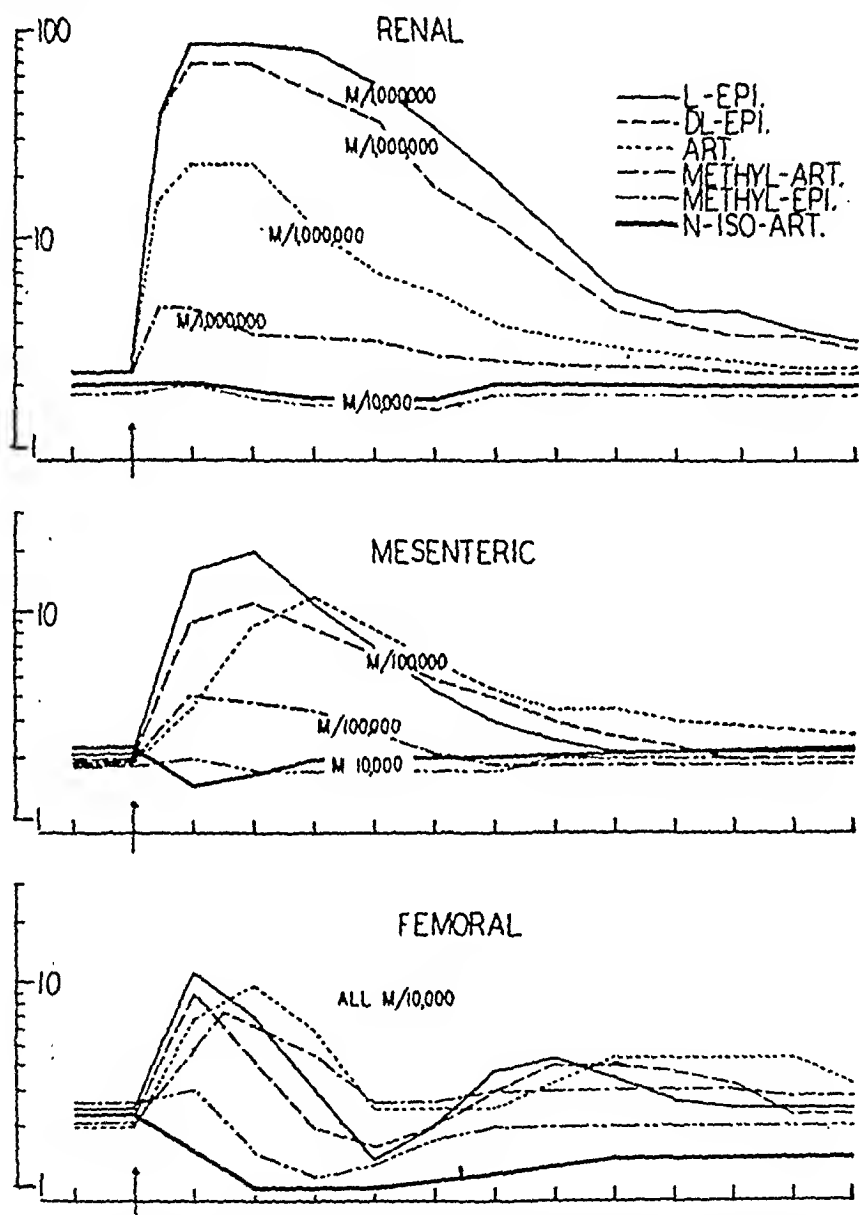


Fig. 1. COMPARATIVE ACTION OF AMINES ON vasomotor resistance in the renal, mesenteric and femoral vascular beds of dogs. Ordinates: VR plotted logarithmically for convenience only; abscissae: time marks at 10 sec. intervals. The amines were injected intra-arterially as 0.1 cc. of the concentration shown. In the case of the renal and mesenteric curves, lower concentrations of *methyl-epi.* and *N-iso-art.* had no appreciable effect on the VR.

were both very active depressors. On the other hand, *art.* and *methyl-art.* were both poor depressors, with the former being the less active in this respect. The usual depressor effects of *Methyl-epi.* and *N-iso-art.* were slightly augmented. In dogs the results were essentially the same, with the exception that *art.* did not lower the pres-

sure in any animal tested. In rabbits none of these amines produced depressor responses after Dibenamine, with the occasional exception of *N-iso-art.*

The relative order of activity of these amines on the vascular receptors can now be summarized as follows (see table 1). On the dilator receptors *N-iso-art.* is the

TABLE 1. EXPERIMENTAL RESULTS. THE MOST ACTIVE AMINE ON EACH RECEPTOR IS NUMBERED 1, THE NEXT MOST ACTIVE 2, ETC.

ADRENOTROPIC RECEPTOR AND METHOD OF EVALUATION	ORDER OF RELATIVE ACTIVITY					
	<i>l-epi.</i>	<i>dl-epi.</i>	<i>art.</i>	<i>methyl-art.</i>	<i>methyl-epi.</i>	<i>N-iso-art.</i>
CARDIOVASCULAR						
Vasoconstrictor						
Renal vasomotor resistance.....	1	2	3	4	5	
Mesenteric " ".....	1	2	2	4	5	
Femoral " ".....	1	3	2	4	5	
Pressor action in cats.....	1	4	2	3	5	
Pressor action in dogs.....	1	3	2	4	5	
Pressor action in rabbits.....	1	2	3	4	5	6
Vasodilator						
Renal vasomotor resistance.....					2	1
Mesenteric " ".....					2	1
Femoral " ".....	3	4			2	1
Coronary dilation.....	2	4	6	5	3	1
Depressor after Dibenamine.....	2	4	6	5	3	1
Myocardial						
Perfused rabbit and cat.....	2	4	6	5	3	1
Intact dog.....	2	4	6	5	3	1
INTESTINAL (inhibitory)						
Isolated rabbit, rat etc.....	1	2	3	4	5	6
Intact dog, cat and rabbit.....	1	2	3	4	5	6
UTERINE						
Excitatory						
Isolated rabbit.....	1	2	3	4	5	6
Intact dog and rabbit.....	1	2	3	4	5	6
Inhibitory						
Isolated rat, cat, rabbit.....	2	4	6	5	3	1
Intact dog, cat, rabbit.....	2	4	6	5	3	1
URETERAL (excitatory)						
Intact rabbit.....	1	2	3	4	5	6?
DILATOR PUPILLAE (excitatory)						
Intact cat.....	1	2	3	4	5	6
NICTITATING MEMBRANE (excitatory)						
Intact cat.....	1	2	3	4	5	6?

most active followed in order by *l-epi.*, *methyl-epi.*, *dl-epi.*, *methyl-art.* and *art.* The relative activity on the constrictor receptors must be interpreted by correlating their actions on both the dilator and constrictor receptors with the relative 'number' of each of these receptors in the vascular bed or species studies. The dilator activity of *l-epi.* and *dl-epi.* is so great that this effect diminishes the net constrictor or pressor effect in those vascular beds or species having many dilator receptors. Therefore

l-epi. is considered to be the most active amine on the constrictor receptors followed in order by *dl-epi.*, *art.*, *methyl-art.*, *methyl-epi.* and *N-iso-art.*

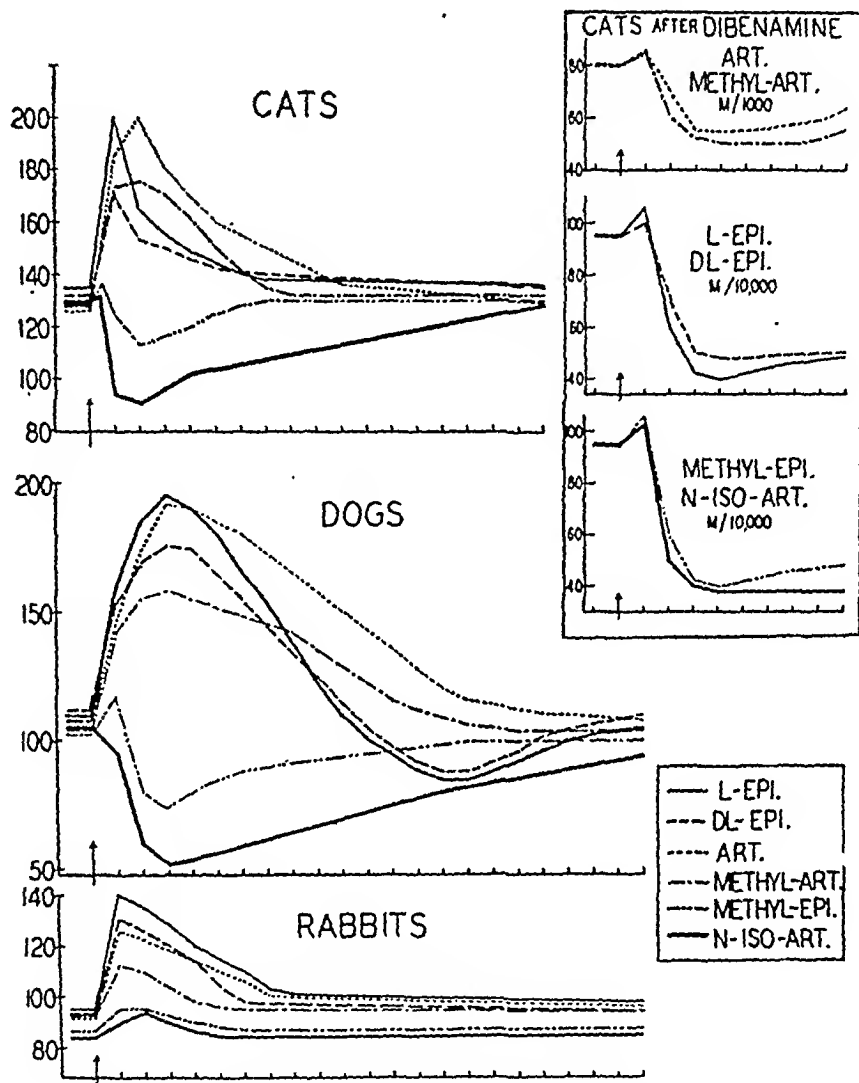


Fig. 2. COMPARATIVE ACTION OF AMINES on mean arterial pressure of cats, dogs and rabbits. Ordinates: pressure in mm. Hg; abscissae: time marks at 10 sec. intervals. The amines were injected intravenously.

Cats: average results from 12 determinations in 6 cats. Dosage, 0.1 cc. M/1000 solution per kgm. **Cats after dibenamine:** average results from 3 determinations in 3 cats. Dosage, 0.1 cc. per kgm. of the concentrations shown. Lower concentrations of *art.* did not produce any depressor responses. **Dogs:** average results from 12 determinations in 8 dogs. Dosage, 0.05 cc. M/1000 solution per kgm. **Rabbits:** average results from 10 determinations in 6 rabbits. Dosage, 0.1 cc. M/1000 per kgm. Larger doses of *N-iso-art.* produced depressor responses.

The relative activity of these amines was determined on both the perfused and intact heart. The isolated heart of the rabbit or cat was perfused with Ringer-Locke solution by the method of Lagendorff. Oxygen containing 5 per cent carbon dioxide was used to aerate the solution and to maintain the perfusion pressure (usually about 100 mm. Hg). Coronary inflow was measured by the rotameter and

the cardiac contractions were recorded with an optical lever system. This method allowed an absolutely simultaneous record of coronary inflow and myocardial activity to be obtained. Figure 3 illustrates the type of record obtained, while table 2 gives the results obtained on 20 rabbit hearts.

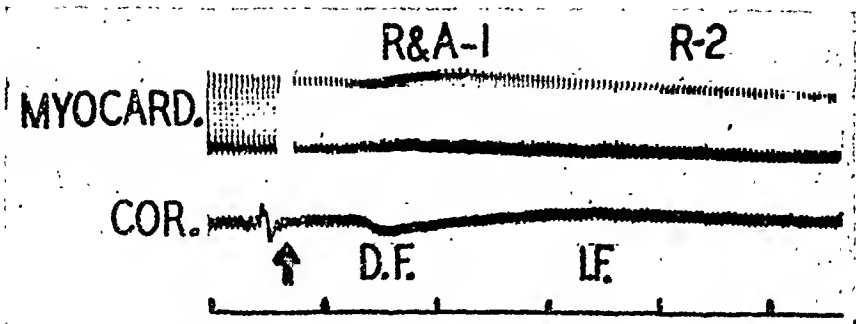


Fig. 3. EXAMPLE OF THE ACTION of the amines on the perfused rabbit heart. Cardiac contractions (myocard.) recorded with an optical lever. Coronary inflow (cor.) recorded with a rotameter. Time marks at 30 sec. intervals. The measurements listed in table 2 were made at the indicated points: R & A-1, the increased rate and amplitude at the point of maximal effect; R-2, the increased rate at a point about 2 min. after the amine administration (a measure of the duration of action); D.F., the decreased coronary inflow due to the increased myocardial activity; and I.F., the increased coronary flow due to the direct action on the coronary vessels. The amines were injected into the coronary inflow.

TABLE 2. COMPARATIVE EFFECTS OF THE AMINES ON THE PERFUSED RABBIT HEART¹

AMINE	INCREASE IN RATE AND AMPLITUDE			CORONARY FLOW	
	R - 1 ^a	A - 1 ^a	R - 2 ^b	D.F. ^c	I.F. ^d
	%	%	%	%	%
<i>N</i> -iso-art.....	101	90	49	11	18
<i>l</i> -epi.....	75	70	26	11	15
<i>methyl</i> -epi.....	62	67	17	13	10
<i>dl</i> -epi.....	57	45	11	18	4
<i>methyl</i> -art.....	40	43	10	16	4
art.....	38	35	0	20	3

¹ Averages obtained from 20 hearts in which each amine was tested in each heart in a dosage of 0.1 cc. M/10,000 solution injected into the coronary inflow. See figure 3.
^a Increase in rate and amplitude at the time of maximum effect.
^b Increase in rate about 2 min. after injection.
^c Decreased coronary inflow measured at point of maximum decrease.
^d Increased coronary inflow measured at point of maximum increase.

Each of the amines increased the rate and amplitude of the cardiac contractions, the relative order of activity being given in tables 1 and 2. Simultaneous with the myocardial stimulation there was a decrease in the coronary inflow which was probably mechanical in origin (29). The flow then returned to or above the control value. Since the coronary flow in the perfused heart is determined by the balance between the myocardial stimulation and the vasodilation, those amines with the least dilator activity would allow the greatest mechanical decrease in flow. In contrast, those amines with the greatest dilator action would allow the least mechanical effect. The

relative order of coronary dilator activity in the perfused heart will then be seen to be the same as described above for the other dilator receptors.

The relative order of activity of these amines on the intact myocardium was determined by two methods. Wiggers (30), Krop (31) and Remington and Hamilton (32) have shown that epinephrine shortens the time of systole (T_s) even though the heart rate may be unchanged. This shortening of T_s which probably results from the myocardial stimulation was also produced by the other amines studied. The T_s was measured on aortic pressure pulse contours of dogs from the beginning of systolic upstroke to the incisura. The apparent order of activity of these amines to shorten the T_s was found to be the same as their relative order on the isolated myocardium.

The blood pressure effects of these amines in dogs pretreated with ergotoxine can be used as a measure of their cardiac action since this sympatholytic agent, in common with dibenamine and Priscol, does not prevent their myocardial actions. Although ergotoxine unmasks the vasodilator effects of the amines it also produces enough vasoconstriction in some dogs to diminish the depressor actions of the amines. This effect allows the amines to produce an increase in arterial pressure in these animals by myocardial stimulation, an effect confirmed by cardiac output measurements by the contour method of Hamilton and Remington (33). The comparative activity of these amines on the myocardium as tested by this method was found to be the same as found on the perfused heart.

The relative order of activity of these amines as myocardial stimulants will be seen (table 1) to be the same as that found for their vasodilator actions. This indicates that the myocardial receptor is related to the vasodilator receptor rather than to the vasoconstrictor receptor.

Intestine. The contractions of the isolated ileum of rabbits, cats and rats were recorded in the usual manner using a 40 cc. muscle chamber and Ringer-Locke solution. Three types of dosage were used: *a*) equimolar concentrations applied to the same segment of the ileum, *b*) equivalent concentrations to the same segment and *c*) equivalent concentrations to different segments from the same animal (only one amine to each segment). The results were the same for each method and for each species and are tabulated in table 1.

The contractions of the intact ileum of anesthetized dogs, cats and rabbits were recorded with a sensitive Hamilton manometer by means of a small, water-filled balloon inserted through a stab wound in the intestinal wall. In all of these animals the arterial pressure was also recorded and in many of them other records such as mesenteric or femoral blood flow, or activity of the uterus or nictitating membrane were also obtained. Only equimolar doses of the amines were compared since the vascular effects of these amines interfered with their intestinal effects. The order of relative activity was found to be the same as that found on the isolated ileum. The most active amine was *l-epi.*, followed in order by *dl-epi.*, *art.*, *methyl-art.*, *methyl-epi.* and *N-iso-art.* This order of activity indicates that the intestinal inhibitory receptor must be related to the vasoconstrictor receptor rather than to the vasodilator receptor.

Uterus. This organ presents a peculiar problem because of its well-known varia-

tions of response to epinephrine. The findings in the literature, and those reported herein, indicate that all uteri have two adrenotropic receptors, one excitatory and the other inhibitory in function, and that the response to epinephrine is determined by which receptor is predominant. The cat uterus, for example, is usually inhibited when nonpregnant and excited when pregnant. The isolated rabbit uterus, on the other hand, is always excited. The intact uteri of dogs, rabbits and humans (pregnant or nonpregnant) exhibit a diphasic response, excitation followed by inhibition.

The responses of the isolated nonpregnant rat uterus (inhibitory) and the rabbit uterus (excitatory) were recorded as described above for the isolated intestine. The comparative effects of the amines on these two species are given in table 1. The order of inhibitory action is seen to be the same as found on the vasodilator and myocardial receptors: *N*-iso-*art.* is the most active, followed in order by *l*-*epi.*, *meth*-*l*-*epi.*, *dl*-*epi.*, *meth*-*l*-*art.* and *art.* The order of excitatory activity is the same

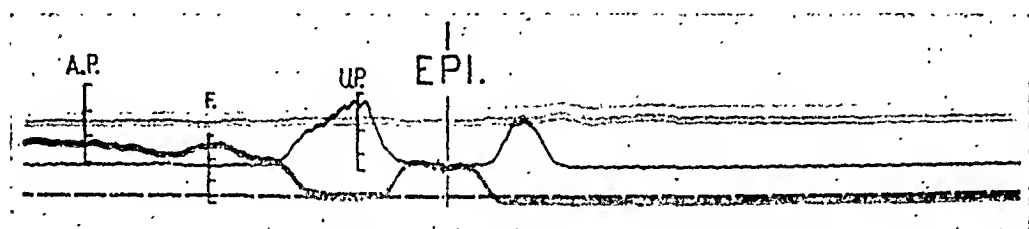


Fig. 4. EFFECT OF INTRA-ARTERIALLY INJECTED EPINEPHRINE on the uterine activity and blood flow. Calibrations: arterial pressure (A.P.) and intra-uterine pressure (U.P.) in units of 50 mm. Hg; uterine blood flow (F.) in units of 10 cc. per min. Time marks at 10-sec. intervals. During the control period the uterus was contracting every 3 to 4 min. At the signal, 0.1 cc. M/1000 *dl*-*epi.* was injected into the uterine artery. Note that during the control contraction the decrease in blood flow occurs simultaneously with the uterine contraction. Following the *dl*-*epi.*, the flow diminished first, then one uterine contraction appeared, followed by complete inhibition. The zero flow and uterine inhibition persisted for more than 30 min.

as found on the vasoconstrictor and intestinal inhibitory receptors, *l*-*epi.*, the most active, followed by *dl*-*epi.*, *art.*, *meth*-*l*-*art.*, *meth*-*l*-*epi.* and *N*-iso-*art.*

When the isolated rabbit uterus was pretreated with Priscol, a sympatholytic agent, the amines lost their excitatory effects and produced only inhibition, with the order of activity being the same as found on the rat uterus. Due to a limited supply, only 2 virgin cat uteri were tested and these gave results identical to those found on the rat uterus.

The responses of the intact uteri of pregnant and nonpregnant dogs, cats and rabbits were recorded as described above for the intact intestine. The results obtained here were difficult to interpret, not only because of the diphasic effects that occurred but also because the muscular effects were complicated by the vascular effects. This can best be described by the following example. The uterine blood flow and the intrauterine pressure were measured simultaneously in a dog a few days post-partum (34). The intra-arterial injection of 0.1 cc. of M/1000 *dl*-*epi.* produced a complete cessation of blood flow lasting about 30 minutes. One uterine contraction was produced and this was followed by complete inhibition also lasting for about 30 minutes (fig. 4.). In contrast to this result, the administration of an equimolar dose of *N*-iso-*art.* produced a slight increase in flow together with a slight inhibition

of activity; these effects lasted only 3 minutes. These results show that the vascular effects so modify the duration of action of the muscular effects (constriction increasing and dilation decreasing) that a true comparison of activity becomes almost impossible. With intravenous injections the arterial pressure changes added still another complicating factor.

The apparent relative activity of these amines on the intact uteri is given in table 1. The order of activity on the uterine inhibitory receptor (derived from the primary effect in the non-pregnant cat and the secondary effect in dogs and rabbits) is seen to be the same as found for the isolated rat uterus. The order of excitatory activity (derived from the primary effect in dogs and rabbits) was the same as described for the isolated rabbit uterus.

Iris and Nictitating Membrane. The comparative effects of the amines on these structures were determined in anesthetized cats. The responses of the *dilator pupillae* were determined by direct observation, using an ordinary 60-watt lamp at 12 inches for illumination. The movements of the nictitating membrane were recorded with a simple string and lever system. Some of the eyes were acutely denervated by section of the cervical sympathetics but this procedure had no apparent influence on the results. In some of the cats the amines were injected into the common carotid to avoid their blood pressure effects as much as possible.

Equimolar and equivalent doses were tested. The relative order of activity of these amines was found to be the same on both structures as shown in table 1. The most active amine was *l-epi.*, with *dl-epi.* about one-half as active, *art.* about one-sixth, *methyl-art.* one-fifteenth, and *methyl-epi.* one-fortieth. Only occasionally did *N-iso-art.* have any effect on these structures and then only when given in a very high dosage directly into the artery.

Ureter. The actions of the amines were compared on the ureter of the anesthetized rabbit. The ureter was exposed at the renal pelvis and cannulated. Saline was perfused under a pressure of about 25 cm. of water, the rate of flow being recorded with a drop counter. The bladder was incised to allow free escape of the perfusate. All of the amines, except *N-iso-art.*, decreased the flow when injected intravenously. The relative order of activity as determined by the amount of flow decrease produced by equimolar doses is shown in table 1. *N-iso-art.* had practically no effect on the flow except when given in very high dosage at which time it increased the flow rate.

The adrenotropic receptor of the rabbit ureter appears to be mainly excitatory in nature and related to the uterine excitatory and vasoconstrictor receptors.

DISCUSSION

The relative order of activity of these sympathomimetic amines on the various adrenotropic receptors is summarized in table 3. There are at least two distinct general types of these receptors. One type of receptor is associated with most of the excitatory functions and with at least one of the inhibitory functions (intestine). The other type is associated with most of the inhibitory functions and with one important excitatory function (myocardium). The relative order of activity of *dl-epi.* and *art.* on the vasoconstrictor receptor is opposite to that previously reported in

the literature. This difference is probably due to the previous methods of comparison, in which the pressor effects only were used as the criteria of activity. The fact that *dl-epi.* is more active than *art.* is more in accord with their relative activities on some of the other excitatory receptors.

Because of the opposite effects associated with each type of receptor, the customary signs, *E* (excitatory) and *I* (inhibitory), cannot be applied. Therefore, for

TABLE 3. SUMMARY OF THE RELATIVE ORDER OF ACTIVITY OF THE AMINES

RECEPTOR	ORDER OF ACTIVITY					
	Most active					Least active
Vasoconstrictor.....	l-epi.	dl-epi.	art.	methyl-art.	methyl-epi.	N-iso-art.
Uterine excitatory....	l-epi.	dl-epi.	art.	methyl-art.	methyl-epi.	N-iso-art.
Nictitating membrane excitatory.....	l-epi.	dl-epi.	art.	methyl-art.	methyl-epi.	N-iso-art.
Dilator pupillae excit- atory.....	l-epi.	dl-epi.	art.	methyl-art.	methyl-epi.	N-iso-art.
Ureteral excitatory....	l-epi.	dl-epi.	art.	methyl-art.	methyl-epi.	N-iso-art.
Intestinal inhibitory...	l-epi.	dl-epi.	art.	methyl-art.	methyl-epi.	N-iso-art.
Vasodilator.....	N-iso-art.	l-epi.	methyl-epi.	dl-epi.	methyl-art.	art.
Uterine inhibitory.....	N-iso-art.	l-epi.	methyl-epi.	dl-epi.	methyl-art.	art.
Myocardial excitatory.	N-iso-art.	l-epi.	methyl-epi.	dl-epi.	methyl-art.	art.

TABLE 4. STRUCTURES OR FUNCTIONS CONTAINING OR ASSOCIATED WITH EACH OF THE TWO TYPES OF ADRENOTROPIC RECEPTORS¹

ALPHA RECEPTOR	BETA RECEPTOR
Vasoconstriction	Vasodilation
Viscera	Skeletal muscle
Skin	Coronary
Nictitating membrane	Viscera (few)
Uterus (excitatory)	Myocardium
Rabbit	Uterus (inhibitory)
Dog	Rat
Human	Cat
Intestine	Dog
Ureter	Human
Dilator pupillae	Bronchi (20)

¹ The following structures or functions have not as yet been completely tested: spleen, *erectores pilorum*, urinary bladder, glands and glycogenolysis.

convenience they have been designated as the *alpha* adrenotropic receptors and the *beta* receptors. Table 4 lists the structures or functions associated with each type of receptor.

This concept of two fundamental types of adrenotropic receptors is directly opposed to the concept of two adrenergic mediators. Epinephrine has all of the chemical and physical properties, and, as shown in table 3, all of the physiological properties necessary to be the only adrenergic mediator. It is the most active substance on the *alpha* receptors and almost the most active on the *beta* receptors. It is, therefore, the one amine which is both the best excitatory agent and the best inhibitory agent

on the effector cells thus far tested. It is fundamentally the most logical substance to be the sympathetic neuro-hormone since all histological and embryological evidence points to the similarity of the adrenal medulla and the adrenergic post-ganglionic nerves.

Cannon and Rosenblueth based their belief in two kinds of sympathin on three fundamental observations (4): *a*) ergotoxine affects released sympathin and injected epinephrine differently, *b*) the combined effect of simultaneously released sympathin from two different sources appears to be greater than expected and *c*) the substance released by the stimulation of the hepatic nerves (liver sympathin) has physiological actions different from those of injected epinephrine. In order to support the theory of a single mediator and two receptors, it is necessary to challenge the validity of the conclusions drawn from these observations.

A comparison of the effects of intravenously injected epinephrine and sympathin released by stimulation of the lower abdominal sympathetics is not valid because of the difference in the manner of delivery of the two substances to the effector cells. A constrictor substance presented intra-arterially, or released directly into a vascular bed by nerve stimulation, tends to hold itself in the periphery by its own constricting action. This can be readily shown by injected epinephrine into the femoral artery while measuring the femoral blood flow. Ergotoxine does not prevent this effect since it, in common with the other known sympatholytics, is not absolute in its sympatholytic or adrenolytic action. Therefore, a large amount of sympathin formed in the periphery by nerve stimulation (the figure reproduced by Cannon and Rosenblueth shows that prolonged nerve stimulation was done) would, even in the presence of ergotoxine, result in contraction of the vessels of that region, which in turn would hold the sympathin and not allow free circulation to other sites. The constriction would therefore be restricted and prolonged. Epinephrine, on the other hand, administered intravenously, would be delivered to all parts of the body, and therefore in low enough concentration to produce vasodilation by selective action upon the adrenotropic dilator receptors.

The apparent difference between sympathins from two sources (the cardio-accelerator and splanchnic nerves) as interpreted by Cannon and Rosenblueth is questionable, since the cardiovascular effects were not considered. The effects (on the nictitating membrane) of the sympathins were separately quantitated against intravenously injected epinephrine. Simultaneous stimulation of the two sources then produced a greater response than expected from the original quantitation. The amount of sympathin delivered to the nictitating membrane is a function of the amount formed at the remote ending and of the blood flow from that region to the membrane. The blood flow was undoubtedly increased during the double stimulation (especially by the cardio-acceleration) and tended to force (against the holding effect described above) more of the splanchnic sympathin to the membrane. This would render the original quantitation invalid. The results may then be explained on the basis of delivery of different amounts of a single substance rather than by a potentiated effect produced by two different substances.

The substance produced by stimulation of the hepatic nerves (liver sympathin) has been the subject of much study. It is evident that liver sympathin is not epine-

phrine. There is, however, no absolute evidence that it is the neuro-hormone (sympathin) or sympathin E. According to Cannon and Rosenblueth, liver sympathin contracts the nictitating membrane, increases arterial pressure, is not 'reversed by ergotoxine' and does not dilate the pupil or relax the pregnant cat's uterus. Further studies by Greer *et al.* (13), and Gaddum and Goodwin (17) have confirmed, in the main, these observations, but have also shown that it does relax the intestine, and may dilate the pupil and relax the cat's uterus.

Liver sympathin is related to epinephrine, and several investigators (9, 10, 13, 17) have suggested that it might be *art.* Euler has obtained a sympathomimetic substance by extraction of mammalian hearts and has suggested (19) that it might be the epinephrine isomer, *methyl-art.* Of the amines used in this study *art.* appears to be the most similar to liver sympathin.

Cannon and Rosenblueth considered the lack of pupillary dilator action of liver sympathin as a paradox, on the basis that this substance was the hypothetical sympathin E. Actually however, the stimulation of the nictitating membrane by liver sympathin is the paradox, if liver sympathin is *art.* It has been shown that *art.* is almost equally effective on the iris and the nictitating membrane. Therefore, an amount of liver sympathin, if it is *art.* that would contract the membrane, should also dilate the pupil. As Gaddum and Goodwin (17) have pointed out, the nictitating membrane is very responsive to many substances other than sympathomimetic amines. It is possible that stimulation of the hepatic nerves could produce small amounts of histamine or acetylcholine which might account for the marked action of liver sympathin on the nictitating membrane.

Assuming that liver sympathin is *art.*, does this amine possess the necessary qualifications to be called sympathin E? The answer must be no, since the results reported herein (based on the racemic forms) demonstrate conclusively that *art.* is not the most active agent on *any* of the excitatory adrenotropic receptors. Just why sympathin from the liver should be chiefly excitatory has never been explained. The source must either be the nerve endings in the liver cells or the endings in the smooth muscle of the hepatic blood vessels. Evidence has been obtained by direct flow studies that epinephrine can produce vasodilation in the hepatic vessels in a manner analogous to its action on other vascular beds (35). Therefore, if there are two kinds of sympathin there is no good reason why an inhibitory substance should not also be formed by hepatic nerve stimulation.

Considering all of the facts available, it is apparent that liver sympathin is a unique product, possibly a modification of the neuro-hormone chemically changed by the liver substance after formation; and that no other substance like it has ever been conclusively demonstrated as being formed by stimulation of other sympathetic nerves. It should therefore be considered as an exception and not be used as the entire basis for the proof of the existence of two kinds of sympathin.

Although little can be said at the present time as to the fundamental nature of the adrenotropic receptor and the difference between the *alpha* and *beta* types, this concept should be useful when studying the various actions of epinephrine, the actions and interactions of the sympathomimetic agents, and the effects of sympathetic nerve stimulation. Use of the terms sympathin E and I should be discouraged, and

the term sympathin should be used to distinguish between the neuro-hormone produced by nerve stimulation and exogenous epinephrine. Fortunately in the case of the cholinergic nerves there has never been any suggestion that there might be two mediators, although both excitatory and inhibitory effects are produced. The diverse effects of the cholinergic mediator, acetylcholine, have always been ascribed to differences in the receptors upon which it acts.

SUMMARY

There are two distinct types of adrenotropic receptors as determined by their relative responsiveness to the series of racemic sympathomimetic amines most closely related structurally to epinephrine. The *alpha* adrenotropic receptor is associated with most of the excitatory functions (vasoconstriction, and stimulation of the uterus, nictitating membrane, ureter and *dilator pupillae*) and one important inhibitory function (intestinal relaxation). The *beta* adrenotropic receptor is associated with most of the inhibitory functions (vasodilation, and inhibition of the uterine and bronchial musculature) and one excitatory function (myocardial stimulation). Racemic epinephrine (and therefore levo-epinephrine which is about twice as active) is the one amine which is the most active on both the *alpha* and *beta* receptors.

The results support the theory that there is only one adrenergic neuro-hormone, or sympathin, and that sympathin is identical with epinephrine. The so-called liver sympathin (the sympathin E of Cannon and Rosenblueth) is regarded as a modified form of sympathin, chemically changed by the liver after formation; and liver sympathin is also considered a unique product and should not be regarded as sympathin E or even sympathin.

The amines were kindly furnished by the following: Drs. K. K. Chen, M. L. Tainter and Melville Sahyun. The heparin was generously supplied by Lederle Laboratories, the dibenamine by Givauden-Delawanna, Inc. and the Priscol by Ciba Pharmaceutical Products, Inc. The author is especially grateful to his colleagues of the Departments of Pharmacology and Physiology, who have been most generous and helpful with their criticisms and suggestions.

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ACTION OF TETRAETHYLAMMONIUM ON CHEMORECEPTOR AND STRETCH RECEPTOR MECHANISMS¹

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TRANSMISSION in autonomic ganglia is interrupted by tetraethylammonium (1), probably by competition with acetylcholine for a 'receptor substance' in the ganglion cells. The specialized receptor cells of the carotid and aortic glomi, formerly thought to be related to autonomic ganglia in ontogenetic origin and in structure, have recently been shown to possess no clear morphologic relationship to ganglion cells (2, 3); but in their responses to numerous drugs the chemoreceptors resemble ganglionic cells very closely. While the physiological stimulus to the chemoreceptor is hypoxia, or perhaps a resultant increase of intracellular hydrogen ion concentration (4), it is well known that these cells can also be excited by acetylcholine (and numerous other quaternary ammonium compounds) and by nicotine, both of which also stimulate ganglion cells, and by the cyanide ion. The present study was undertaken to determine whether chemical excitation of the chemoreceptors, like that of ganglion cells, would be modified by tetraethylammonium. Stretch receptor mechanisms were studied for comparison

METHODS

All experiments were performed on dogs anesthetized with thiopental followed by sodium barbital. Arterial blood pressure was recorded with a mercury manometer. Respiratory rate and amplitude were recorded by means of rubber bellows tied about the chest and attached to a tambour; in a few cases minute volume of expired air was recorded by a 100-liter spirometer. In some experiments, the carotid bodies were denervated and the vagi sectioned to demonstrate that the doses of cyanide used produced effects mediated almost entirely by chemoreceptor mechanisms. Tetraethylammonium (T.E.A.) was administered at varying rates by continuous intravenous infusion, and the test drugs acetylcholine chloride, nicotine hydrochloride, sodium or potassium cyanide, lobeline sulfate, and veratridine hydrochloride were given by single or repeated intravenous injection. Acetylcholine was given only after atropinization.

RESULTS

Typical respiratory responses to acetylcholine, nicotine and cyanide before and during infusion of tetraethylammonium are illustrated in figure 1. Acetylcholine

Received for publication May 3, 1948.

¹Supported by a grant from the Life Insurance Medical Research Fund.

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in a total dose of 4 mgm. caused a brief increase of respiratory rate, amplitude and minute volume, accompanied by a primary fall of arterial pressure (presumably because of incomplete atropinization) and a secondary moderate rise due to ganglionic stimulation of sympathetic vasoconstrictor pathways. The hyperpneic and pressor responses to twice this dose were abolished by T.E.A. infusion. The depressor phase persisted, since the action of acetylcholine on effector cells is not prevented by T.E.A. Similarly, the action of nicotine was blocked; during infusion of T.E.A., 0.8 mgm. failed to alter respiration or blood pressure, although 0.2 mgm. caused a vigorous hyperpnea during the control period. The inhibition of the response to nicotine was

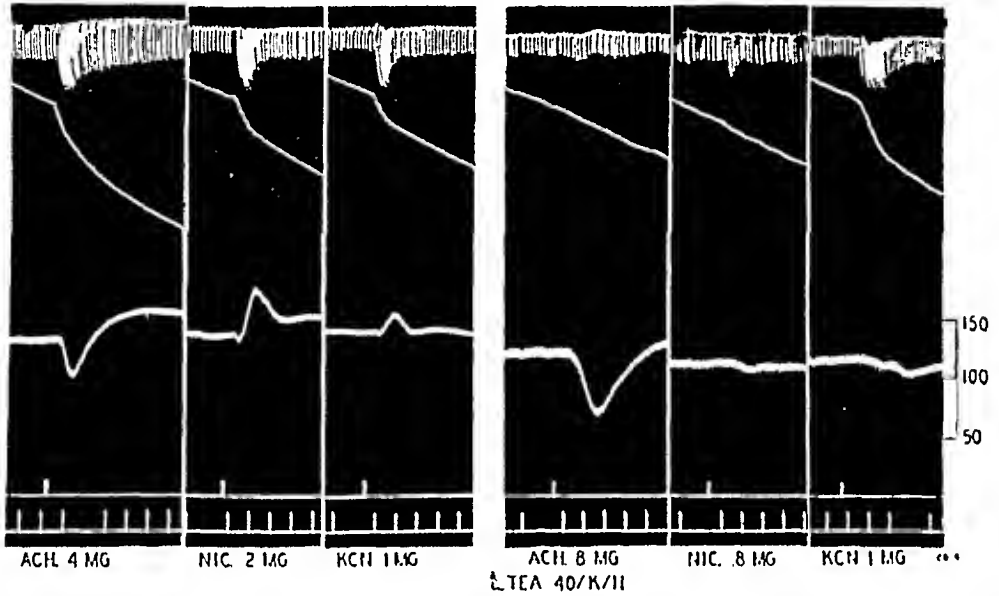


Fig. 1. Dog, 9.3 kgm. Tracings, top to bottom: respiration (tambour), respiratory minute volume (spirometer), arterial pressure, signal, time in 10-sec. intervals. Continuous infusion of T.E.A. during last three segments.

shown not to be due to development of tachyphylaxis to this drug, for responses returned after cessation of the T.E.A. infusion. The action of cyanide, however, was not blocked by T.E.A. Similar results were obtained in five additional animals. In one experiment lobeline sulfate (Lilly) produced responses which, like those of nicotine, were blocked by T.E.A.

Blockade of the carotid body responses to acetylcholine and nicotine, like blockade of ganglia, is apparently competitive in nature, for a level of T.E.A. administration which blocks the minimal dose of these agents effective during the control period can be overcome by the injection of larger doses, and the responses to the larger doses of acetylcholine and of nicotine can again be blocked by increasing the infusion rate of T.E.A.

Since T.E.A. fails to block the action of cyanide, it was expected that it would also fail to prevent the hyperpneic response to hypoxia induced by the respiration of a mixture of 5 per cent oxygen and 95 per cent nitrogen. This was confirmed as illustrated in figure 2A. The respiratory responses to hypoxia, to hypercapnea (10%

carbon dioxide) and to asphyxia were essentially unaltered by high infusion rates of T.E.A.

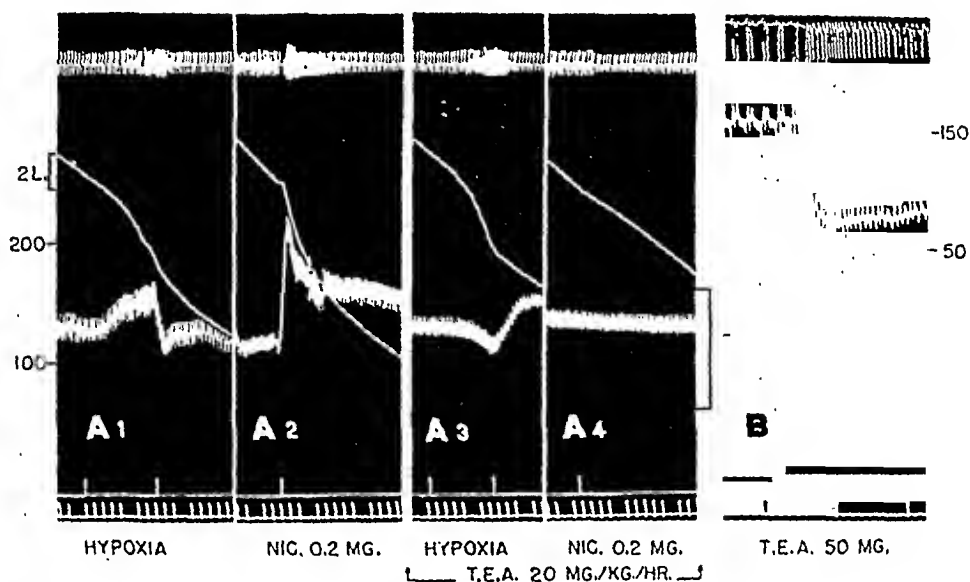


Fig. 2A. DOG, 10.0 kgm. Tracings as in fig. 1. Between segments 2 and 3, manometer was shifted; scale at left applies to first two segments. The bracket at the right of segment 4 extends from 0 to 100 mm. Hg and applies to segments 3 and 4. Hypoxia: 5% O_2 , 95% N_2 ; Nic.: nicotine hydrochloride.

Fig. 2B. DOG, 11.2 kgm. Tracings: respiration (tambour), arterial pressure, signal, time (in minutes). Pressure scale at right in mm. Hg.

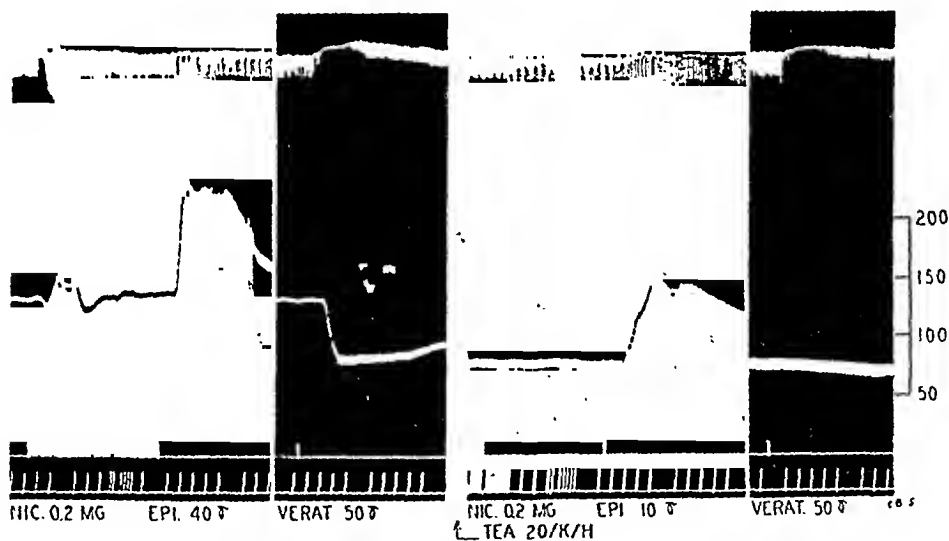


Fig. 3. DOG, 8.3 kgm. Tracings: respiration (tambour), arterial pressure, signal, time, 10 sec. Continuous infusion of T.E.A. during last two segments.

The action of T.E.A. on the pressoreceptors could not be judged from cardiovascular responses to alteration of intracarotid pressure, for the efferent autonomic pathways involved in such reflexes are blocked by the drug. Respiratory responses were therefore studied. Intracarotid pressure was reduced by bilateral carotid

occlusion or by injection of glyceryl trinitrate, or increased by the injection of epinephrine. The increased respiratory rate caused by reduction of carotid pressure was not prevented by T.E.A.; indeed, a relative tachypnea usually occurs as the pressure falls from the injection of T.E.A. itself (fig. 2B). Similarly the slowing or cessation of respiration induced by epinephrine persists during infusion of T.E.A. (fig. 3). It may be concluded that the pressoreceptor response to mechanical stimulation is not affected.

The responses of pulmonary stretch receptors also appear to be unaffected by T.E.A. Administration of the drug by single dose or continuous infusion causes a reflex acceleration of respiration induced by diminished arterial pressure; it does not cause the slowing and the increase of amplitude which result when the Hering-Breuer reflexes are interrupted by vagal section. Further evidence on this point was obtained in a dog in which the chest was opened and artificial respiration administered at a minute volume which just failed to suppress spontaneous respiratory efforts. In this state, respiratory movements were synchronous with the strokes of the pump. Cyanide and lobeline caused a great increase of amplitude of the respiratory efforts, but because of the continuing influence of the vagal reflex mechanism the rate remained equal to that of the pump. During infusion of T.E.A. the response to lobeline was blocked, but the response to cyanide was unaffected, and the thoracic excursions remained synchronous with the pump, proving that the pulmonary vagal receptors were unaffected by T.E.A.

Veratridine has been shown to cause a reflex bradycardia, hypotension and apnea (5). The site of the afferent endings initiating the cardiovascular responses has been localized to cardiac terminations of the vagi, while the respiratory response is effected by stimulation of vagal endings in the lungs (6). As in the case of responses to pressoreceptor stimulation, the cardiovascular responses to veratridine are blocked by T.E.A. through interruption of the efferent pathways. Apnea, however, is not prevented by T. E. A. (fig. 3); it may be concluded that these unique vagal receptors, like the stretch receptors of the lungs and the carotid and aortic areas, are unaffected by T.E.A.

DISCUSSION

Histologic studies of the carotid and aortic bodies (2, 3) demonstrate the presence of columns of epithelioid cells, presumably but not certainly of neural origin. These cells are closely approached, and perhaps entered (2) by neurofibrils. The few ganglion cells which have been described are not believed to have any functional significance in the chemoreceptor function. Assuming that the epithelioid cells are indeed modified neurons, specially developed for a chemosensitive function, the impulse aroused in these cells must be transmitted to the fibrils of the afferent nerves. Whatever the origin of these cells, and whatever their relationship to ganglia may be, it is conceivable that transmission across this unique synapse could be effected by acetylcholine. This would account for the sensitivity of these structures to acetylcholine and related substances. According to the concepts of Gesell and his collaborators (4, 7), the normal physiological stimulus, hypoxia, could act by decreasing intracellular pH and thus potentiating the transmitter. If such a cholinergic synapse

exists, it is difficult to explain the action of T.E.A., for the latter, by blocking the synapse, should prevent responses to cyanide and hypoxia as well as those to acetylcholine and nicotine. However, if Nonidez' view is correct, that at least some of the nerve fibrils enter the receptor cells, it would be expected that such an intracellular 'synapse' would be inaccessible to T.E.A., while exogenous acetylcholine could, of course, still be blocked. In the light of our present incomplete knowledge of the functional anatomy of the glomⁱ, no more extensive speculation can be made; it can only be stated that the chemoreceptors respond to acetylcholine, whether or not this plays a physiological rôle, and that the response to acetylcholine, but not to hypoxia or cyanide, can be blocked by T.E.A.

Large doses of T.E.A. cause death in the anesthetized dog by respiratory depression. Respiration ceases while somatic neuromuscular transmission remains unimpaired; the mechanism is thus obviously not curariform. Since the carotid body response to hypoxia, which plays an important rôle in maintaining adequate ventilation of animals under barbiturate anesthesia (8), is not blocked by T.E.A., the respiratory depressant action of this drug is probably exerted centrally.

The activity of pressoreceptor mechanisms is apparently unaltered by T.E.A. This was expected, since the stimulus for such stretch receptors, and for those in the lung, is mechanical. The failure of T.E.A. to block the cardio-pulmonary vagal afferents suggests that these may be stretch receptors whose sensitivity is altered by the veratrum alkaloids, rather than chemoreceptors stimulated by these drugs. The latter possibility cannot, of course, be eliminated, for there is no reason to suspect that T.E.A. could compete in such a chemoreceptor mechanism.

SUMMARY

1. Tetraethylammonium blocks the action of acetylcholine, nicotine, and lobeline on the carotid body. The effects of cyanide and of hypoxia on the chemoreceptors are not blocked.

2. T.E.A. does not prevent the activity of the stretch receptor mechanisms in the carotid sinus and aortic arch, or in the lungs.

3. T.E.A. prevents the reflex cardiovascular responses to veratridine, by interrupting ganglionic transmission in the efferent limb of the arc; it does not block the afferent vagal endings in the thorax as judged by the persistence of the respiratory response.

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PANTOTHENIC ACID AND CARBOHYDRATE METABOLISM IN THE RAT

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IN A study of factors affecting survival in adrenalectomized rats, we had observed (1) that, with adequate amounts of sodium chloride, the addition of calcium pantothenate to a pantothenic-acid deficient diet resulted in a remarkably long period of survival. In fact, survival with calcium pantothenate plus sodium chloride was significantly longer than that obtained when either cortical extract plus salt or desoxycorticosterone acetate (DOCA) plus salt were added to the deficient diet. We are now investigating the manner by which pantothenic acid may operate to effect the prolonged survival of the adrenalectomized rat.

As pantothenic acid is known to play a rôle in carbohydrate metabolism (2-9), and as the adrenal cortex is associated with carbohydrate metabolism, it seemed possible that the prolonged survival of the adrenalectomized rat fed calcium pantothenate might be due to some effect of pantothenic acid on carbohydrate metabolism. Before proceeding to study the adrenalectomized deficient animal, it was necessary to investigate the effect of pantothenic acid on the carbohydrate metabolism of the intact rat. In order to provoke a severe disturbance in carbohydrate metabolism, one series of experiments has been done in which the effect of pantothenic acid was studied in the alloxan-treated rat.

EXPERIMENT I

Procedure

Fifty-two rats of the Long-Evans strain, bred in the laboratory, were used in this experiment. The rats were divided into three groups. *Groups A* and *B* (total of 46 rats) were placed on the pantothenic-acid deficient diet (2) when the animals were 34 days old and were continued for 29 to 38 days on the deficient diet before treatment with alloxan was initiated. These rats received a solution of 1 per cent sodium chloride as drinking water. The rats in *group A* (27 rats) were continued on the deficient diet after the injection of alloxan. The rats in *group B* (19 animals) were given 4 mgm. of calcium pantothenate daily in addition to the deficient diet. The calcium pantothenate was begun either 4 or 5 days prior to alloxan injection (13 animals) or on the day of injection (6 animals). The animals in *group C* (6 rats) were fed the Nu-Chow diet and were injected with alloxan when they were 49 days old. All animals of *groups A, B* and *C* were injected intraperitoneally or subcutaneously with alloxan

Received for publication May 5, 1948.

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monohydrate in a dose of 17.5 mgm. per 100 grams of body weight after a preliminary fast of 17 or 24 hours. The alloxan was made up in a 5 per cent solution in distilled water. The degrees of glycosuria, polyuria, polydipsia and weight loss were used as an indication of the severity of the diabetes. The animals were not considered diabetic unless the glycosuria persisted for at least 7 days. The animals rendered diabetic were observed for an average of 30 days. Three of the deficient and 5 of the supplemented diabetic animals were given varying amounts of protamine zinc insulin subcutaneously in an attempt to control the diabetes. Five other deficient, 1 supplemented, and 2 animals on the Nu-Chow diet, all of which were diabetic, were subsequently bilaterally adrenalectomized under ether anesthesia from the 7th to the 27th day following alloxan treatment.

TABLE I

GROUP	DIET	NO. OF RATS	PANTOTHENIC ACID BEGUN	HOURS OF FAST PRIOR TO ALLOXAN	ROUTE OF INJECTION	RATS DEVELOPING DIABETES		MORTALITY OF RATS DEVELOPING DIABETES		MORTALITY OF RATS NOT DEVELOPING DIABETES		TOTAL MORTALITY OF THE GROUP	
						No.	%	No.	%	No.	%	No.	%
A	Panto- thenic acid deficient	11	—	17	I.P.	4	36	3	75	6	86	9	82
		16	—	24	S.C.	5	31	0	0	1	9	1	6
		27	—	—	—	9	33	3	33	7	39	10	37
B	Panto- thenic acid supple- mented	6	On day of alloxan	17	I.P.	2	33	0	0	2	50	2	33
		5	4 or 5 days	17	I.P.	3	60	1	33	0	0	1	20
		8	prior to alloxan	24	S.C.	1	12	0	0	3	43	3	38
		19	—	—	—	6	32	1	17	5	38	6	32
C	NuChow	6	—	17	I.P.	2	33	0	0	2	50	2	33

Results

The results of Experiment I are summarized in table I.

Length of fasting period and route of alloxan injection. A fast of 17 hours followed by an intraperitoneal injection of alloxan did not cause any difference in the development of diabetes in the deficient rats when compared with a 24-hour fast followed by a subcutaneous injection. The mortality rate experienced with the former combination, however, was considerably higher. In the pantothenic acid supplemented group, the 17-hour fast followed by an intraperitoneal injection produced more diabetic animals but there was little difference in the mortality rate.

Incidence of diabetes. The incidence of diabetic animals seemed to be little affected by the nature of the previous diet; since approximately only one third of the animals in each group exhibited glycosuria for more than 7 days. The alloxan was given in a single dose and, as many workers advise repeated injections, this undoubt-

edly influenced the incidence of glycosuria. In the deficient animal, repeated injections are hazardous but may still be necessary to consistently produce the diabetic state.

Severity of diabetes. The extent of the glycosuria varied considerably in the individual animals and a spontaneous remission of the glycosuria also occurred in some animals. The absence or presence of pantothenic acid in the diet did not appear to affect the glycosuria or the degree of polydipsia and polyuria.

Response to insulin. Injections of protamine zinc insulin decreased or abolished the glycosuria regardless of whether the rats were on the deficient or supplemented diet. Relatively large doses of insulin were tolerated, one animal receiving as much as 4 units in a single dose.

Response to adrenalectomy. The 5 diabetic animals on the deficient diet which were bilaterally adrenalectomized became aglycosuric and died between the 6th and 13th post-operative days. Of the 2 diabetic animals on the Nu-Chow diet which were similarly adrenalectomized, one died within 24 hours, the other lived 8 days with only traces of sugar in the urine. A single diabetic animal on the supplemented diet became aglycosuric and continued to gain weight after adrenalectomy for 17 days at which time it was sacrificed.

EXPERIMENT 2

Procedure

Thirty-four Long-Evans rats on the Nu-Chow diet were injected subcutaneously with alloxan monohydrate (5 per cent solution in distilled water) in a single dose of 17.5 mgm. per 100 grams of body weight at the age of 87 days. An additional dose of 10 mgm. per 100 grams in a 2½ per cent solution of distilled water was given subsequently to any resistant or only slightly glycosuric animal. The 12 rats thus made diabetic were then paired off on the basis of body weight and degree of hyperglycemia. There were thus 6 pairs of animals, one of each pair receiving the pantothenic acid deficient diet and the other receiving the same daily amount of the deficient diet as consumed by the paired feeding mate and, in addition, receiving 4 mgm. of calcium pantothenate daily. Both groups received a solution of 1 per cent sodium chloride as drinking water. Daily food, water consumption and urinary output were measured. The degree of glycosuria was determined by using Clinitest tablets. The animals were observed for 32 days. Blood sugar levels were determined on the 11th and 22nd days of the experiment in each animal, using the method of Polis and Sortwell (9a).

Results

The results of the paired feeding experiment are summarized in table 2. The blood sugar changes in the 2nd, 3rd, 4th, and 5th pairs of animals did not appear to be significant, considering normal variations and the error in the method. The deficient animal of *pair number 1* showed a 30 per cent increase in the level of blood sugar while the supplemented rat showed a 102 per cent increase. In *pair number 6*, the deficient animal showed a 40 per cent decrease while the supplemented animal showed a 16 per cent decrease. The change in weight revealed rather marked individual

variability but no consistent trend in either the deficient or supplemented animals. The average daily urine volume was, likewise, quite variable with no consistent trend in either group. The urine volumes, for the most part, were quite high. The supplemented rat in *pair number 6* had an average daily urine output of 258 cc., or more than 2.5 times its body weight.

TABLE 2

PAIR NO.	DIET	1ST FASTING BLOOD SUGAR	GLYCO- SURIA ON DAY OF 1ST F.B.S.	2ND FASTING BLOOD SUGAR	GLYCO- SURIA ON DAY OF 2ND F.B.S.	% CHANGE IN FAST- ING BLOOD SUGAR	% CHANGE IN WT.	AVERAGE DAILY URINE VOL.
		mgm. %		mgm. %				cc.
1	Deficient	90	1+	117	4+	+30	+12	76
	Supplemented	105	4+	212	2+	+102	-4	223
2	Deficient	126	4+	135	4+	+7	+4	84
	Supplemented	116	1+	123	0	+6	+14	13
3	Deficient	114	1+	116	4+	+1	-3	105
	Supplemented	144	2+	122	4+	-15	+3	194
4	Deficient	126	2+	108	4+	-1	-7	158
	Supplemented	127	0	123	tr.	-3	+19	19
5	Deficient	123	2+	116	0	-6	+12	29
	Supplemented	101	4+	91	0	-10	+2	51
6	Deficient	304	3+	183	4+	-40	-9	108
	Supplemented	209	2+	176	4+	-16	-29	258

DISCUSSION

The results of the experiments indicate that alloxan diabetes produced in the manner described above is an extremely variable syndrome. Complete spontaneous remission of the glycosuria may occur and such inherent variability would tend to mask any effect of the absence or addition of pantothenic acid to the diet.

The strikingly low blood sugars in some of the 'diabetic' animals in the paired feeding experiment may be partially accounted for by the rather prolonged fasting period. Many investigators (10-14) have described, in addition to the customary pancreatic changes, histological lesions in the convoluted tubules of the kidney of varying severity after alloxan treatment. Lazarow (14) found that the blood non-protein nitrogen paralleled the renal damage. Bennett (15) found no difference between the diabetic and nephrotoxic dose of alloxan in rats in the Long-Evans strain. It is quite possible that the glycosuria of alloxan diabetes may be due in part, at least in the early phase of the diabetes, to renal damage.

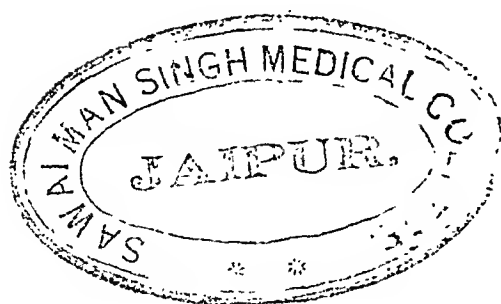
SUMMARY

Alloxan-treated young black rats exhibit a diabetes of inconstant severity which responds to insulin and which is ameliorated by bilateral adrenalectomy. The glycosuria during the early phase of the diabetes may be partly due to the renal damage. No significant effect on the diabetes was noted due to the addition or absence of pantothenic acid in the diet. The inconstancy of the diabetes produced by alloxan makes it of questionable value as a method of studying the rôle of pantothenic acid in carbohydrate metabolism.

This research was aided by a grant from the National Vitamin Foundation.

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